

Who will regulate the bankers?

That financial institutions should be trustworthy is a necessary condition for their continued sponsorship of innovation. Banks, in other words, need laws.

NOT so long ago, banks were the epitome of stability in society. Grave sober-suited men (there were hardly any women) would move around marbled banking halls confident that even if they were not much liked, they were at least respected, veritable pillars of society. Now all that has changed. On both sides of the Atlantic and in the Far East as well, banks have taken to going bust. Last year, there were more banking failures in the United States than in any previous year since the great depression. During the same year, in Britain, the Bank of England moved quickly to rescue from impending collapse the Johnson Matthey Bank. Why has there been this rash of banking failures? Do they matter? And, if so, what can be done to prevent them?

A bank is an institution that makes a business of looking after other people's money. It will borrow money from one group of people and lend that same money on to other people. Its lenders are recompensed by the payment of part of the interest the borrowers are required to pay; the remainder goes to the owners of the bank, a reward for their time, trouble and expense. A banking system is an essential ingredient of an industrial society; if people had no choice but to keep personal wealth in gold, hidden perhaps in a mattress, there would be no means by which one person's wealth could finance another's innovation. The social function of the banks is to make some people's personal wealth accessible to others who may make better use of it, the creation of credit as it is called.

The only other way of financing innovation so far devised entails the embodiment of most wealth in the state, and the subsequent investment of the same funds by civil servants, as attempted in countries such as the Soviet Union. In the West, technology as it has become is as much the creation of the banking system as of technologists. Certainly the collapse of the banking system would bring technology to a halt. One of the great conceptual developments of the past few years has been the discovery that all financial institutions that take in money from some people and lend it to others have exactly the same economic function as those formally called banks.

Fraud

Obviously, such a system is open to abuse, most simply by the crude device in which the bankers convert depositors' money to their own use, paying enough interest to keep the victims happy and being prepared to decamp when all the funds have gone. In most places, bankers who operate in this fashion are considered criminals, and may finish up in gaol. Others may run into trouble innocently, by lending their deposits unwisely, either because they too readily share their borrowers' optimism (which partly accounts for the troubles of banks in the US Middle West that, two years ago, found themselves over-committed to oil exploration) or because of unexpected changes in the economic climate. Between these extremes of fraud and innocence, the unhappy experience of the past few years has revealed a wealth of ingenious schemes by which bankers, in the pursuit of profit, can run into trouble. Lending money to borrowers unlikely ever to repay the funds may seem profitable until the period of the loan runs out.

Banks and other financial institutions are also vulnerable to

fraud, by individuals or even other institutions. Embezzlement, the device by which bank officers siphon off deposits for their own use (often with the conviction that it can be repaid) was once the most common reason why some bankers would be drummed out of their community; it has become a means by which financial institutions can be busted, as with those sections of the Lloyds insurance market in London that have been affected by the scandals of the past few years. Among the tricks that institutions have learned to play on each other are the use of the same collateral to raise more than a single loan, one of the devices by which the Florida company EMS Securities kept itself afloat while insolvent until, last year, it collapsed, bringing down a string of Ohio banks with it. The collapse of the Singapore stock market in the past few weeks has been brought about by yet another device, essentially one by which present credit was traded against future obligations (which could not be met).

Fear

What now worries Western governments, and should worry the rest of us, is the fear that the financial troubles of the past few months will get out of hand. Governments are in a nasty fix. Having recognized that all financial institutions are banks of a kind, and recognizing the importance of banks as a means of stimulating economic activity, they have moved quickly to remove regulations preventing one set of financial institutions from competing with another. The technology the banking institutions have helped to stimulate has brought further complications; dealing in financial securities (debt obligations) has become literally a 24-hour business. The rewards of success are greater than ever. The risks of failure are similarly unprecedented, as is the suspicion that a large part of the debt hanging over the markets, the obligations of the debtor nations of Latin America in particular, may be worth not much more than the paper (or silicon) on which it is recorded. And the much-publicized misdemeanours of the heirs of the black-suited men of a previous generation has raised the question whether bank officers can be trusted with the future of the world's prosperity.

So enthusiasm for deregulation is being overlaid by enthusiasm for further regulation. In Britain, a curious situation has arisen. Since the early 1970s, successive governments have rightly encouraged changes of the rules by which institutions of different kinds must live. Competition among financial institutions has indeed increased, while London has strengthened its place on the international scene, roughly half-way between Tokyo and New York. Two years ago, the stock market (in exchange for the government's agreement not to pursue a complaint that its internal rules were restraints on competition) agreed to dispense with the most irksome of these restraints, the rules regulating those who may trade in stocks. The result now is that there are powerful and newly created financial institutions, many of them offshoots of conventional banks, waiting for the day next October, called the "big bang", when competition is to be free. The only certainty is that not all the hopeful institutions can hope to succeed, or even survive.

The dangers in such a prospect should be plain for all to see. The social function of the London stock market, like that of



other stock markets, is to provide a means of buying and selling the debt obligations of governments and commercial companies, essential if lenders are to be persuaded to lend. But stockbrokers, with the help of banks of different kinds, also function as a means of bringing new money into the system, in which respect they function as if they were banks in the more familiar sense. The London stock exchange (like others) sets out to regulate the securities that may be bought or sold and even the terms on which trading is allowed, yet there is no means by which lenders (called investors) can be protected against their own poor judgement (nor should there be). But the unprecedented circumstances in which, later this year, much enlarged stockbroking firms will be fighting each other for business that will not expand overnight to match their ambitions are plainly full of risk; the London stock market (like others) maintains a fund to compensate investors who lose money when stockbrokers fail, but there are plainly arithmetical limits to what the collective power of commission-men can do to safeguard the assets belonging to those on whose behalf they deal.

That is only one of the reasons why the British government should be more active in the regulation of its financial institutions. The issue has been bubbling away for two years, since the publication of the report on the steps needed to safeguard investors by Sir Jim Gower. The government has already created a Securities Investment Board whose function will be to superintend the working of financial institutions other than banks (looked after by the Bank of England), the stock market (which superintends itself) and the Lloyds insurance market (which purports to do the same). While the new board will require all those who take in money to be registered, it will ordinarily agree that groups of like-minded institutions should look after themselves. Similarly, the supervision of ordinary banks by the Bank of England is to be overseen by another new board, but one nominated by the Bank of England itself. Before this intended legislation becomes law, it may easily be amended. It should be toughened, and for a simple reason.

However well-deserved may have been the reputation for probity of past bankers, the sanctions they used to apply to keep each other on the straight and narrow path no longer apply. A fraudster who knows that he can move easily from one financial centre to another will not be much deterred by knowing that he will be disinvited from the better golf clubs in the place at which his activities are first discovered. The financial community in Britain is nevertheless offended that there should be talk of explicit external regulation of its affairs, while the government is over-inclined to listen to these arguments. But in the long run, there will be no alternative to explicit regulation. The British government had better bite that bullet now, before too much damage is done. Financial institutions are too important to the economy to be left solely in the hands of financiers. □

OPEC is not yet dead

The oil producers will now be less powerful unless the oil consumers invite further discomfort.

OPEC, the Organization of Oil Exporting Countries, continues to exist, but only just. That is the simplest reading of the meeting at Geneva early in December, when the member states decided that they could no longer by *fiat* fix the world price for crude oil. This development has been on the cards for at least the past two years, for as long as OPEC's fixed price has been greater than the price at which oil was to be had on the Rotterdam spot market, and while OPEC members have themselves been selling oil at prices below the official price. So is it possible now to celebrate the end of an awkward period in the affairs of the industrialized West and then to forget?

That is what complacency suggests, but complacency is a poor guide. OPEC came into being a quarter of a century ago because the oil-exporting countries quite rightly tumbled to the fact that

they were being exploited. In the 1950s, crude oil was being taken from Saudi Arabia at less than one US dollar a barrel, of which less than a half found its way to the government of that kingdom. During that period, in the United States, it was necessary artificially to prop up the price of domestically produced oil by means of cumbersome sponsored devices such as the Texas Railroad Commission, a federally sponsored cartel. But OPEC showed its strength only in 1969, when Libya cancelled the concessions previously let to multinational oil companies, and began selling its own oil to those prepared to buy it. From that point, the developments of 1973, when the official price of OPEC's crude oil was multiplied roughly five times, were inevitable. The further but smaller increases of price in 1979, ostensibly compensation for inflation in oil-consuming states, were a proof that OPEC was still in a strong position.

What has happened since was also predictable. In the oil-consuming states, the intensity of energy consumption has decreased steadily and dramatically, partly because of greater efficiency but also because of the changing pattern of goods manufactured in the industrialized West. It could easily come about that energy consumption, and oil consumption in particular, will increase again if economic growth accelerates, but many old inefficient ways have probably gone for good.

But none of this implies that OPEC, too, has gone for good. In the long run, the present period of OPEC's weakness, brought about by the declining demand for oil by the industrialized economies and the success of exploration for oil (at OPEC's high price) elsewhere in the world, has been accentuated by the urgent need of some oil-producing governments to maintain their oil revenues at the high levels to which they have been over-fortunately accustomed. Iran and Nigeria, importunately compelled to sacrifice their long-term interests for the immediate need for cash, have done as much to weaken OPEC as the compensatory market mechanisms at work elsewhere.

For the years immediately ahead, the course of events is predictable enough. Now that oil prices have fallen (to not much more than \$25 a barrel or \$175 a tonne), the incentives to exploration will be diminished (but not much), economic activity in the industrialized economies should be stimulated (but, again, not spectacularly) and the price of oil will find a new level at which the interests of the producers and the consumers are balanced. But further ahead, early in the next century perhaps, there will be a different setting for the oil market. By then, no doubt, there will have been further important discoveries of oil in parts of the world still unexplored, so that the potential membership of OPEC will have been enlarged or at least changed (for some oil producers will also have used up their reserves). Similarly, there may then also be now unexpected developments in energy technology that could have transformed the pattern of consumption. But what will stand out, at the turn of the century, is that Saudi Arabia will almost certainly remain in the unique position it occupies at present, as the country most richly endowed with plentiful reserves of crude oil that differs in the most radical respect from that available elsewhere — its production cost is very much less.

This is the sense in which December's events do not spell the end of OPEC. Even if in the years ahead it is destined to become a not particularly congenial dining club, there is every reason to expect that the market for petroleum will change in such a way that Saudi Arabia will again be in a position to play a leading part in sharpening OPEC's teeth. For the oil consumers, the lesson should be clear. Whatever new discoveries there may be, and whatever the fluctuations of the price of crude oil, there are the strongest strategic reasons for strengthening the influences that have induced the energy economies of the past few years. Artificial restraints are inappropriate, as are subsidies of any kind. The better rationalization of industrial production internationally may be more important. Nobody wants OPEC to become a tyrannical influence on the world economy again, but the safeguards are in the hands of the oil consumers. □

Japanese public health

New campaign launched against hepatitis B

Tokyo

JAPAN'S Ministry of Health and Welfare launched the world's first public hepatitis-B immunization campaign on 1 January. Funds have been provided to immunize 10,000 babies each year identified as being at risk by the presence of hepatitis Be (HBe) antigens in their mothers.

The go-ahead for the programme follows by a few weeks the commercial release of hepatitis-B vaccines from two domestic manufacturers, the Kitasato Research Institute and Green Cross. A third manufacturer, the Chemo-thero Therapeutic Institute, will have a similar product available in May. Licences to produce the vaccines were granted in April after clinical trials were completed. The vaccines are derived from the plasma of hepatitis-B virus carriers inactivated by heat and formalin treatment and are essentially the same as those licensed in the United States in 1982.

Carrying out the programme is very expensive. Each of the three injections required costs around 5,000 yen (US\$25) and this is added on to the high cost of identifying mothers whose babies are at risk. One thousand million yen (\$5 million) has been allocated by the government for the first year of the campaign. Hepatitis B is a major public health problem in Japan not only because of the seriousness of hepatitis itself but also because it can lead to cirrhosis and cancer of the liver. Hepatitis-B virus infection has been linked to more than 80 per cent of all cases of primary hepatocellular cancer: the immunization campaign may thus fairly claim to be the first that might wipe out one form of cancer. Mother-to-child transmission of hepatitis B is the most important route of production of hepatitis B carriers; 85–90 per cent of babies borne to HBe-antigen-positive mothers will have become carriers by the time they are three months old. Clinical trials of the immunization method on 3,000 babies in Japan have already shown that transmission can be virtually wiped out.

Japan's selective immunization campaign is not, however, likely to serve immediately as a model for other countries. First, Japan is one of the few affected nations wealthy enough to bear the costs of immunization and with the expensive procedures for testing for HBe antigen established as routine at health centres. Again, although the overall frequency of hepatitis-B carriers is, at 2.6 per cent (rising to 5 per cent in some areas of Kyushu), quite high compared to Europe and North

America, it is still sufficiently low that identifying those at risk is cheaper than a mass immunization campaign.

In the developing countries of Asia, the problems are of a different magnitude. In both Taiwan and Korea, around 15 per cent of the population are carriers and in

mainland China around 10 per cent. Chinese officials say that the cost of vaccine would have to fall by one to two orders of magnitude before any mass immunization campaign could be contemplated.

Hopes for a fall in costs lie with the genetically engineered vaccines now just coming onto the market. The vaccines, produced by the Pasteur Institute and Genentech in mammalian cells and by Merck in yeasts, are still very expensive. But the potential market is huge and Japanese companies are in the race to produce cheap vaccines. **Alun Anderson**

Australian science

Radical shakeup planned

THE Australian Science and Technology Council (ASTEC), the world's most prolific source of recommendations on the management of publicly supported research, has advocated radical changes in the organization of the Commonwealth Scientific and Industrial Research Organization (CSIRO). The recommendations are put forward by a subcommittee of ASTEC under its chairman, Professor R.O. Slatyer of the Australian National University, Canberra.

In a report* to the federal prime minister published at the end of last year, ASTEC advocated the replacement of the present top management of the organization, called the "executive" and consisting of three full-time and two part-time members, by a board of seven part-timers and a single full-time official, which would be the source of research policy for the organization. The most startling aspect of this proposal is that the full-time official, to be called the Chief Executive, would be appointed by the board (rather than by the government) and would be liable to dismissal by the board.

According to the report, the chief purpose of the proposed changes would be to ensure that the lines of responsibility for research policy and for management within CSIRO are unambiguous. The Slatyer committee has considered but rejected arguments that CSIRO should be split up into separate organizations responsible for research in different fields, on the grounds that Australia needs a strong central (and flexible) research organization but the committee urges that CSIRO's strategy should be more closely directed towards the needs of existing and emerging industries, saying that CSIRO programmes exclusively concerned with basic research should be hived off to other institutions, most probably universities, "perhaps in conjunction with a scheme to identify national centres for research in which a number of universities might participate".

The starting point for many of the Slatyer

committee's proposals is a sense of regret that the last reform of CSIRO eight years ago has not produced the benefits expected of it. The report says that CSIRO's advisory council has not been able to influence policy as intended (and that it should be abolished), and that the same conclusions apply to the state and territory committees that at present formally have a role in guiding research policy. The Slatyer committee is especially regretful that the 1977 reform that grouped CSIRO research laboratories (called divisions) into three institutions, each with a separate director, has blunted rather than strengthened the links between top management and the divisions.

Although the Slatyer report does not say so explicitly, its recommendations reflect the evolution of Australian research institutions and in particular the increasing strength of the universities in research. But the sustained emphasis in the Slatyer report on the need that CSIRO should concentrate its efforts and resources on providing assistance for industry (including the traditional industries of agriculture and mining) will, if accepted, bring about a considerable change both in the pattern of research at many of the well-established research divisions and in the ethos of CSIRO as Australia's chief employer of those bent on a career in research.

On the last point, the report says that CSIRO should give up its now common practice of employing most of its new recruits on indefinite contracts (there is also at present a one-year probationary contract) and that it should instead switch to a system in which an initial period of employment on a short-term contract would be the general rule, with an understanding that only a proportion of those thus hired would win permanent appointment. The committee says this pattern would, among other things, serve to provide opportunities for postdoctoral scientists, especially those returning from abroad. □

*Future directions for CSIRO. Australian Government Printing Office, Canberra.

Remote sensing

US agencies blamed for muddle

Washington

AN outspoken study of the "crisis" in the 13-year-old US Earth remote sensing programme by the National Academy of Sciences has concluded that US pre-eminence in the field is now seriously threatened by "not-so-benign neglect". The academy says that substantially increased government resources will be needed if the potential of the technique is to be realized, and knocks some agency heads together for letting the area fall into disarray.

The academy's report emphasizes that the present situation is urgent. The only civilian Earth remote-sensing satellite in service, Landsat 5, is expected to fail before private industry can launch a replacement. No improvement is likely unless the government increases the \$250 million ceiling it has placed on remote sensing while the programme is being transferred to the private sector.

Although negotiations with an industrial consortium to take over the programme are at an advanced stage, there are still no definite arrangements for the future, and during the probable hiatus in the US service, the French company SPOT (Système Probatoire d'Observation de la Terre) can be expected to secure a large part of the market for remotely-sensed Earth data although customers would prefer continuity of data. SPOT's data will be available and will have some technical advantages.

The academy's report* was produced by its research arm, the National Research Council (NRC), under the chairmanship of George Harter. The report has been more than two years in preparation and seems to have engendered some controversy: an earlier draft by a specially appointed committee was rejected by NRC's Space Applications Board, which then set about producing a better version. The work of the earlier committee is acknowledged only in passing.

NRC is enthusiastic about the ultimate potential of data sensed from space, while pointing out that data must in future (not as at present) be made available to users in processed form if they are to be saleable products. Oil companies, which now use remotely-sensed data for prospecting, have spent years and large research budgets in discovering how to use it. (Oceanographic remote sensing, according to NRC, should remain within the government as a "public good" service.)

Although NRC supports the government's basic strategy of turning Earth remote sensing over to the private sector while keeping meteorological satellites under its own wing, it is harsh in its criticism of how the strategy was carried out.

NRC stresses that technical problems are no part of the reason why the programme has done so badly. Rather, parochialism is to blame: the programme fell between the twin stools of the National Aeronautics and Space Administration (NASA) and the National Oceanic and Atmospheric Administration (NOAA).

Although the two agencies worked together for many years on remote sensing, after NOAA was officially placed in charge of the programme and NASA faced a budget crisis in the early 1980s, NASA threw the ball over to NOAA, which was apparently unready to catch it. Part of the reason, according to NRC, was NOAA's disadvantageous and often-criticized bureaucratic niche within the

Department of Commerce. NRC accordingly recommends that NOAA be found a more suitable home.

Even if NOAA stays where it is, however, NRC is not so pessimistic as to believe the two agencies could never again work together constructively. NRC wants to abandon the present arrangements whereby institutional rather than technical considerations dictate what sensors are flown on a given spacecraft, and proposes a single coordinated effort, with NOAA remaining in charge of applied and market research for Earth data, and NASA providing technical support for the development of new sensors, for example. Finally, NRC tells the two agencies in future to allow each other to use space on their platforms and also to make this available to industry.

Tim Beardsley

**Remote Sensing of the Earth from Space: A Program in Crisis (National Academy Press, 1985).*

French science

CNRS faces political change

SHOULD the Centre National de la Recherche Scientifique (CNRS), the principal French research council, be split up? This seems to be the question of the month in right-wing scientific circles in France, where the general election due in March is expected to return a government of the right. It is difficult to establish exactly where the idea comes from, although *Le Monde* picks on "sources close to Jacques Chirac", the mayor of Paris, expected to become France's next prime minister.

CNRS has had to contend with some criticism of late. The council is certainly big, supporting more than 10,000 academics in disciplines from physics to history, and its multidisciplinary nature has given it considerable flexibility in meeting the challenge of new sciences such as materials, microelectronics, robotics and biotechnology. But in responding to these new goals and in its policy of "overture", opening up the hallowed halls of academic institutions to the public, to industry and to other research bodies, CNRS "has changed profoundly" over the past 3-4 years, says its director-general Pierre Papon.

In so doing CNRS may have created enemies, particularly among more traditional academics used to the old somewhat feudal continental system of professorial fiefdoms that were once prevalent in France. CNRS has attempted to cross these barriers where they still exist — Papon still feels it necessary to preach the values of the British and US departmental system — and has also used its budgetary muscle, in concert with the ministry of education, to concentrate resources on priority areas. The prospect of a right-wing government has given some of those affected a chance to complain, and there is

a growing clamour in France for a return of power to the professor, at the expense of that of the science political apparatus such as CNRS. A fragmentation of CNRS into disciplinary subsections would clearly serve this cause well.

To confuse matters, however, the present research minister in the socialist administration, Hubert Curien, speaking just before Christmas at a prize-giving at CNRS, was ambiguous in his reply to criticisms of the centre. He described the criticisms as "a typical French exercise in self-flagellation", and said that if CNRS did not exist, he would have to invent it; but he also said that "one must not be deaf to criticism" and talked of "a new mode of scientific organization" in "networks" of projects rather than isolated grants.

In the circumstances, one might expect M. Papon to feel somewhat isolated and exposed, but he finds no policy conflict with M. Curien. "Networks", for example, correspond to his own concept of interdisciplinarity. And moreover Papon was re-appointed for a second three-year term of office in October. He fully intends to continue his work under a new government, "unless it shifts policy by 90 or 180 degrees". Also, Papon's appointment was made by the President of France, and François Mitterrand does not face re-election until 1988. Papon also believes that "no responsible government could contemplate a dismemberment of CNRS", or retreat from the present government's policy of harnessing science for the economy, of which he himself has been a major architect. By the middle of the year, when a new government will be preparing its budget and policies for 1987, he should know if he is right.

Robert Walgate

Japanese communications

What band-rate for psi?

Tokyo

WHEN it comes to new methods of telecommunication, Japan's Ministry of Posts and Telecommunications believes in looking at even the long shots. The ministry's fanciful new "Research Committee on Future Telecommunications Media" is, among other things, to look into the prospects for telepathy as a communications medium.

The new eight-member committee has been established on the recommendation of a private advisory panel to the Minister of Posts and Telecommunications. It will be headed by Shigemichi Sonoyama, vice-president of the National Space Development Agency, and to add weight to the panel's discussions, another of its members is Tadahiro Sekimoto, president of the giant electronics company NEC.

Japan is not, of course, the only country in the world to think of the potential of telepathy; it has long been rumoured that the Soviet Union is taking the subject very seriously. But perhaps this is the first time that a posts and telecommunications ministry has taken up the idea. True to Japan's vigorous commercial spirit, the ministry has in mind practical application. An attractive feature of telepathy is that transmission is instantaneous, or so some claim. Dr Ootani, a psychologist at the Self-Defence Forces University, will be the principal scientist to investigate "superhuman abilities", although anyone with such abilities might be expected to avoid research panels and the possibility

of being turned into a kind of living telephone exchange.

Telepathy will not be the only new telecommunications medium to be investigated. One professor from Kanagawa University will take a look at the potential of neutrinos. And another professor from Tohoku University will assess gravity waves, even though ministry officials



agree that their existence is scarcely more certain than that of telepathic waves. Backing up their investigations are experts on brain physiology, electrical communication and computer intelligent management systems.

The committee is expected to hold four meetings by the spring for an initial exchange of views. Although the hopes of practical application of the new media might seem very far off, the ministry says it is not "too early to start study".

Alun Anderson

Red greens on Yellow River?

A BELIEF is growing in China that the proposed hydroengineering works on the Yangtze (Yellow) river will "spoil the scenery", the New China News Agency reports. But an unnamed official of the Yangtze river valley planning office maintains that construction of the proposed hydroelectric power station, which will substantially raise the water level in the Yangtze gorges, will not greatly harm the scenery. In fact the authorities are looking forward to improved amenities and, according to a planning office spokesman, "some new scenery will emerge".

The purpose of the project is threefold. The hydroelectric plant will have a capacity of 13 m kWh, said the spokesman, and will supply power to cities as far away as Beijing, Tianjin and Tangshan in the north, Shanghai in the east, Canton in the south, and Sishuan in the west. The new, higher water level will submerge more than 90 per cent of the dangerous shoals in the three gorges, thereby reducing hazards to navigation and increasing the number of

vessels that can safely be carried on the middle Yangtze and the Chuan river, with a substantial saving in the cost of transport.

At the same time, flood control measures in the three gorges will substantially reduce the risk of flooding to the Dongtin Hu, Poyang Hu and Jiangnan plains, densely populated areas which are China's commodity grain and cotton base. At present, more than 1 million people, the spokesman said, are needed to protect the existing dykes during the flood season each year, and according to archives going back to the end of the Han dynasty, some 2,000 years ago, there was major flooding until the beginning of this century in one year out of ten.

What form Chinese concern over the three gorges has taken is unclear. There is no suggestion of mass protests, but anxiety is clearly sufficiently acute and widespread for the planning office to find it necessary to issue a statement to reassure the public.

Vera Rich

Space science

British head for French lab

FRANCE is building a new £4-million laboratory for space sciences at the Université de Paris Sud (Orsay), and has attracted a leading British space scientist as its first director.

But Dr Alan Gabriel, now head of the space and astrophysics division of the Rutherford Appleton Laboratory near Oxford, who will direct the new Institut de Physique Spatiale d'Orsay (IPSO), says he does not feel part of a brain-drain. "I don't want to be negative about British space science", he said. "It's not true to say things are much better in France than they are here." The new British National Space Centre for the coordination of the UK space effort "offers most exciting possibilities" and it was "not at all" true to say British space science is not well-enough supported. Moreover space science is international, and Gabriel expects much cooperation between IPSO and his British colleagues.

However, Gabriel implies that he feels more cramped at the Rutherford Appleton Laboratory than he expects to be in Paris. Gabriel's present division of around 150 is merely one part of a 1,500-strong multidisciplinary laboratory; in Paris, IPSO will be no bigger than the space and astrophysics division but it will be independent, except for some support from the research councils and the university. "I shall be able to play a more creative role", Gabriel says.

At the new Paris laboratory, two-thirds of the staff will come from an existing French group housed in an overcrowded old laboratory in nearby Verrier-le-Buisson. The Laboratoire de Physique Stellaire et Planétaire has experiments on both the European and Soviet Halley probes (Giotto and Vega).

The new IPSO will also form a focus for a number of space science groups now scattered around the Paris area, and will take advantage of the proximity to LURE, the Orsay synchrotron radiation source, for calibration of optical instruments from the X-ray region to the visible.

IPSO's initial research programme will concentrate on solar physics, infrared studies of the interstellar medium to shed light on mechanisms of star formation and Solar System studies including dust, comets and asteroids. (France is planning for a mission to the asteroids either within the European Space Agency or bilaterally with the Soviet Union.) Gabriel is also interested in developing a capability at IPSO for X-ray astronomy. He expects IPSO to become one of the major centres for space sciences in France.

Robert Walgate

Argentine science

Slow return to openness

Buenos Aires

ARGENTINE science is recovering slowly from the traumas of the past decade. In contrast with 1973, on the eve of the return of Peron, and 1979, after the military had finally repressed the opposition, it is evident that the morale of the people has been uplifted and that there is a strong desire for political stability under democratic rule.

The severe repression by the military dictatorship afflicted all scientists, depriving many of their jobs and some of their lives. One biochemist, apparently considered too outspoken, had a bomb planted in his house and telephone threats which compelled him to leave his university and to move with his family to a remote part of Argentina.

Another outspoken biochemist became a victim of the mindless repression apparently only because he was Jewish. His home was visited one day at five in the morning by men in plain clothes, who had come to arrest him. Fortunately, his wife's mother answered the door and screamed out that the men were thieves. So he escaped with his pregnant wife and young children over the roof tops.

The Israeli government, to its honour, acted as a Scarlet Pimpernel by providing passports for such refugees, irrespective of their religion; a general escape route was organized with the cooperation of the Belgian airline Sabena.

The exodus of Argentine scientists during the repression and their enrichment of the culture of other countries has been documented by Budiansky (*Nature* 311, 201; 1984). Few of those expatriates have yet returned, probably because they cannot afford to; salaries in the universities and government institutes remain depressed. Industrial salaries are relatively remunerative, three or four times those of academics. The low salaries in the state sector (see Budiansky's article) and the general economic malaise are professional people's chief preoccupation.

In the aftermath of the repressions, demands for retribution continue, but scientists are divided. Some, partly out of self-interest or envy, are accusing those who prospered under the military of criminality; others, including many who suffered under the despotism, support the call for a "full stop" to recrimination.

In the universities, a commission is examining the credentials of all professors appointed during the past 20 years, when the normal procedures for appointment were suspended. This is time-consuming for university staff and probably diverts attention from the need to renew the resources of the universities so as to meet the challenges of the future.

Argentina, in common with the developed countries, has greatly expanded its university system in the past two decades. (Cordoba, founded about 1650, is probably the oldest university in the Western Hemisphere.) Although the old universities are still dominant, such extra resources as there have been seem to have been concentrated on the new universities, so that there has been little renovation of the older universities, which are now dilapidated and inadequately equipped to strengthen or maintain their position as centres of science.

Nevertheless, scientists are pressing on in reasonably good spirits, although they seem no way out of the impasse caused by Argentina's economic problems. Subscriptions to journals have been cancelled wholesale; support for assistants has to come from professors' personal salaries. Buildings are in an appalling state, but all new building schemes have been frozen by the government.

There is a lack of campuses in the older universities, so that departments are widely scattered, and thus isolated from interaction with others and prevented from sharing equipment, libraries and overheads generally. Apparently, the authorities in the past have been suspicious of campuses as foci of political action by students. Surely such fears of criticism could be abandoned by the democratic government, even if the concentration of resources in campuses will have to be a long-term aim.

One move towards the concentration of resources was the inauguration of research centres by the national research council, CONICET. Buildings, staff and equipment were funded on a modest scale, but again these are dispersed and isolated. They have successfully encouraged good basic science, but now the government intends to transfer them from CONICET to the universities, which leaves researchers fearing that their centres will be reduced to a state of academic deprivation. Integration of the centres, with protection of their resources, is essential if their work is to expand.

In the biosciences, it is striking that, as elsewhere, the emphasis is now heavily on technology. The size of the Argentinian agricultural industry offers much scope for biotechnology to improve plant and animal crops, and eventually to exploit the arid regions and their solar energy. As in Brazil, 10–15 per cent of ethanol must be added to motor fuel to promote the exploitation of sugar by fermentation. Health care products are being developed through innovation in biotechnology. For instance, the Polichaco pharmaceutical company has announced a contract worth

US\$3 million to supply to Brazil diagnostic reagents for pregnancy testing. Excellent molecular biology towards characterizing the virus of Argentinian haemorrhagic fever is being done at the University of La Plata, in laboratories which in Britain would be condemned as unsuitable. With resignation, the researcher says, "This shows that expensive facilities are not essential for success in molecular biology".

The government has set up a committee to develop a policy for biotechnology, and many groups seem to be competing for access to any funds that may be made available. But the government is still searching for a formula to define biotechnology. Yet a policy is needed urgently because of the momentum of research in this field elsewhere in the world and to offer the Argentinian scientists the opportunities they deserve.

Academic scientists have even begun to entertain the idea of obtaining support from industry and other entrepreneurs, but this approach may need legislation; the universities have traditionally prevented academics from having links with industry.

The transfer of technology from university to industry is beset with difficulty. In the fermentation field, despite its rapid expansion to produce ethanol, there is a marked reluctance by industry to take advantage of scientific innovation which Argentine scientists are developing, even in ethanol fermentation.

Remedies may yet emerge from the changing political climate. Both the radical party government under Alfonsín and the recent Austral currency reform seem to be winning confidence. It is a pity that the Falklands (Malvinas) problem remains unsolved. The slogan "The Malvinas islands belong to Argentina" remains conspicuous in public places, even though serious comment recognizes the historical realities and the need for flexibility in Argentinian policy.

There is also a new spirit of cooperation in South America. Chile and Argentina have settled their territorial disagreement and, in the past weeks, there have been agreements between Argentina and Brazil and between Chile and Peru on friendship and cooperation.

The lack of normal relations between Britain and Argentina continues to affect trade. For instance, the supply of radioactive chemicals from Britain has been made difficult. One molecular biologist has resorted to making his own labelled nucleotides, with a cost saving of over 80 per cent on his reckoning if technician's time is discounted. There is an historical affinity between Britain and Argentina that surely requires Britain to settle its differences with Argentina with chivalry and thus to help ensure that despotism never returns there again.

S.J. Pirt

Laboratory animals

US protection act approved

Washington

SHORTLY before its Christmas break, the US Congress passed legislation supported both by animal welfare campaigners and by researchers' organizations designed to improve standards of care for laboratory animals. The Improved Standards for Laboratory Animals Act requires painful experiments to be justified to a committee that includes at least one disinterested member of the public charged with ensuring that pain and distress are minimized.

The new act was introduced by Senator Robert Dole (Republican, Kansas) and Representative George Brown (Democrat, California) with 136 co-sponsors. Animal welfare workers, chiefly the Society for Protective Animal Legislation, worked for two years with researchers' organizations to find acceptable compromise provisions. The bill as passed was supported by, among others, the America Society for the Protection of Cruelty to Animals, the Humane Society of the United States, the American Physiological Society and the Association of Professors of Medicine.

The chief effect of the new act is to require the creation of Institutional Animal Committees that must include one external member; at present, many but not all institutions which carry out research on animals have such committees. The committees will be required to conduct semi-annual inspections of research facilities and to report violations of regulations to institutional and federal authorities. The Secretary of Agriculture is empowered to promulgate regulations on care, treatment and practices to minimize pain and distress, but not on the conduct of experiments.

The new act also requires institutions to provide training in the humane treatment of animals, encourages alternatives to animal testing where possible and requires principal investigators to certify that alternatives have been considered. Exercise for laboratory dogs, use of anaesthesia with paralytics and limitations on repeated major operations on the same animal are also specified.

A new information service at the National Agricultural Library is established under the act, in order to minimize unintended duplication of experiments, and both federal and private laboratories are subject to its provisions. Although the act makes clear that experimental protocols will be respected, it is required to suspend federal support if an agency determines that animal care in a particular project has been persistently unacceptable.

Tim Beardsley

Bhopal disaster

India blames Union Carbide

New Dehli

INDIA's official report on the events leading to the Bhopal disaster last year says that about half a tonne of water had entered the tank containing methyl isocyanate (MIC), thus confirming the findings of Union Carbide. But the report says that the chemical reaction leading to a catastrophic increase of temperature was caused by contamination of the tank by metal ions, which acted catalytically.

The report is the work of a 16-member committee led by Dr S. Varadarajan, the chemist who is now the secretary-general of the Department of Scientific and Industrial Research. It specifically disputes Union Carbide's claim that entry of large quantities of water alone was responsible for the ghastly accident on 2 December 1984, saying that the runaway reaction leading to a rapid rise of temperature and pressure in the storage tank could have been triggered by water only if the MIC inside the tank had also been contaminated with metallic impurities.

The report, by implication, rules out sabotage, alleged by Union Carbide, and instead puts the blame on the US company for its choice of unsuitable corrodable materials for the piping thought to have been the source of the contamination.

Varadarajan's committee reached its conclusions on the basis of experiments carried out on a second undamaged storage tank at Bhopal. The experiments included heating MIC alone and reacting it in the presence of water, chloroform and traces of ferric salts and sealed containers at temperatures up to 250 degrees centigrade. These simulations yielded all the twelve or so different types of solid residues found inside the damaged tank.

Based on these experimental data and an actual inventory of the tank residues, it was estimated that 12,087 kg of MIC and 595 kg of chloroform (used as a solvent) reacted with 512 kg of water, producing enough heat to vapourize the remaining 28 tonnes of MIC in the tank. The report says that the escaped gas also included 80 kg of ammonia and 1.25 tonnes of carbon dioxide.

The Varadarajan report has incidentally put to rest the controversy over whether the gas that leaked was MIC, cyanide or both. Union Carbide had from the beginning insisted that cyanide was not present, but Indian doctors who carried out autopsies on gas victims suspected otherwise. The report agrees with Union Carbide on the basis of the experimental evidence it has collected.

The most important finding of the Varadarajan committee relates to the role of water. Union Carbide maintains that the runaway reaction was due to the addition

of large amounts of water to MIC containing chloroform. The Indian report says that ingress of 500 kg of water would at best have consumed four tonnes of MIC and raised the tank temperature to just 70 degrees centigrade, below the boiling point of MIC (at the safety valve pressure). The report also says that chloroform could not have catalysed the reaction below 200 degrees centigrade, whence the conclusion that some other catalyst must have been involved.

From the products of tank residues, calculated heats of chemical reactions and the extent of bulging in the tank, the committee estimated that the tank reached a temperature above 250 degrees centigrade. According to the report, only "the onset of metal-ion catalysed polymerization of MIC" could account for this rapid increase of temperature.

The Varadarajan committee says that the metallic contaminants (iron and sodium) came from corroded pipes and the tank itself and catalysed the trimerization of MIC. Subsequent entry of water hydrolysed the MIC, producing heat and carbon dioxide and allowing "trimerization to take off with explosive violence".

In support of this theory, the report lists iron, sodium, chromium and nickel among the metallic contaminants found inside the exhumed tank. Sodium was present at a level of 60 p.p.m., equivalent to 25 litres of 5 per cent sodium hydroxide. According to the report, the contaminants most probably came from tank accessories made of materials such as carbon steel and copper.

The source of sodium is identified as the vent gas scrubber. One source of iron was found to be the tank itself, probably released as ferric iron as a result of corrosion caused by hydrochloric acid formed by the hydrolysis of phosgene.

Exactly how the contaminants and half a tonne of water came to enter the tank, the report can only guess. Indirectly, the report rules out sabotage by the argument that while it may be easy to pour water into the tank, a saboteur would not know the role of metallic contaminants.

The report concludes that the gas leak would have been avoided had the 42 tonnes of MIC been stored in several small steel drums. "The needless storage of large quantities of MIC for long periods, as well as insufficient caution in design, choice of materials for construction and provision of measuring and alarm instruments, together with inadequate controls on systems of storage and quality of stored materials as well as lack of necessary facilities for quick and effective disposal material exhibiting instability led to the accident."

K. S. Jayaraman

AIDS in the human brain

THE causative agent of acquired immune deficiency syndrome or AIDS (variously known as LAV, HTLV-III or ARV virus) is akin to the lentiviruses¹. It would appear, on current estimates of AIDS virus prevalence in the US population (about 0.5 per cent), that between 10^5 and 10^6 persons may develop lentivirus-like encephalopathies during the next fifteen years. When other animals are infected with similar lentiviruses (maedi-visna virus in sheep), the disease is progressive and fatal, with an incubation period of one to six years. The duration of AIDS encephalopathies already known in humans is not well established but may range from one to 14 years.

The similarities between the two diseases and their causative agents are established experimentally. Both of them, RNA retroviruses, are integrated into the genome of the host's cells as proviral DNA. Efforts to treat the viral DNA must include protection for the host's DNA, a task of which we are at present incapable. Both AIDS virus and the lentiviruses possess the properties of "antigenic drift", lessening the likelihood of a vaccine as we now understand vaccines. Thus, the burden of disease control falls on epidemiology and prevention.

The implications of such a large number of men, women and children with progressive disease for the economy, health care system and emotional state of society are unprecedented. The scientific necessities, the need to develop strategies for dealing with a new type of human infection and producing a lifetime viraemia pose scientific puzzles of dizzying proportions. Even if my estimates of clinical neurological disease are in error by one or more orders of magnitude, the tragedy is still shocking, especially as it comes on top of the already manifest deaths due to AIDS immune suppression. If AIDS is a lentivirus disease, the immune-suppressed AIDS patients may be only the tip of the iceberg.

The threat of AIDS as a lentivirus disease should motivate politicians, public health workers and scientists to establish a

unified plan for AIDS prevention on local, national and international scales. Since the mechanics of transmission of AIDS-lentivirus are known, the great risk associated with anal intercourse, promiscuous heterosexual encounters, blood to blood contact and the use of shared drug injection paraphernalia must be explained in the most specific terms to all levels of society. Those already infected must be included in the strategy. Universal blood testing should not be ruled out. If AIDS is a lentivirus epidemic, to err on the side of caution would be prudent.

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1. Seale, J.R. *J. R. Soc. Med.* **78**, 613–615 (1985).

Earnshaw's theorem

SIR—This theorem is not often mentioned in scientific journals. It denies the possibility that an electric charge can be held stable solely under the electrostatic influence of other electric charge. It was used in a recent letter (*Nature* **317**, 208; 1985) to refute a case put earlier by Berezin (*Nature* **315**, 104; 1985). It is as well to keep in mind that the Reverend Samuel Earnshaw developed his theorem with an eye to the constitution of the aether as a medium comprising a structured system of electric charges, separated by what, presumably, would be regarded as a truly void state of the vacuum. The theorem fails if the charges permeate a charge plenum or continuum having a charge density, because displacement can then be subject to a linear restoring force rate, owing to interaction with this continuum.

We have now come to accept that the vacuum medium does have some rather special characteristics and a possible structure, so it is not unlikely that it comprises electric charges permeating a charge plenum, notwithstanding the Earnshaw theorem.

If this is the case, then the theorem cannot even be applied without some reservation when considering the mutual stability of charge in matter. The support for the structured vacuum is enhanced by the related theoretical derivation of the fine-structure constant in terms of the geometrical features of an electrical charge system neutralized by a charge continuum. A value of the fine-structure constant in matching accord with its measurement at the level of one part in ten million has recently been reported from such analysis (*Phys. Lett.* **110A**, 113; 1985).

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Price of star wars

SIR—R. V. Harrowell (*Nature* **317**, 470; 1985) criticized the proposed Strategic Defense Initiative on the grounds that if it is successful it will supposedly release deadly amounts of unexploded plutonium into the atmosphere.

Let us compare the radioactivity of this plutonium with that of the radioactive material already present in the air. According to that arcane source of data, *The Handbook of Chemistry and Physics* (66th edition), the atmosphere masses 5.136 hexillion grams, its mean relative molecular mass is 28.966, and the molecular fraction of radon is one part per hexillion. If we combine this information we see there are 0.18 moles of radon in the atmosphere. The half-life of radon is 3.82 days and that of plutonium is 24,110 years; radon is therefore 2.3 million times as radioactive as plutonium. The equivalent of 400,000 moles (about 100 tons) of plutonium is already present in the air in the form of radon. We can compare that with the mass of 5,000 warheads at 10 kg of plutonium each, that is, 50 tons.

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Metric system

SIR—There is a further problem to be considered in the controversy about ambiguities in metric notation, affecting the acquisition of mathematical concepts by school children.

In the days of Imperial measures, areas used to be denoted in units of square inches, square feet, etc. Such quantities were usually written in the form: "16 square inches" or "16 sq. ins". This was naturally read by the child as "sixteen square inches", and was quite unambiguous.

Nowadays it is common to denote areas in the metric system as follows: "16 cm²" which is naturally read as "sixteen centimetres squared". A very understandable confusion then arises in a young mind between "16 centimetres squared", which is just acceptable as a way of expressing 16 cm², and "sixteen centimetres square" which can only mean 256 cm².

The meaning of "sixteen square centimetres" is thus obscured, and is not easy to clarify in verbal terms.

The fault arises, of course, from the linguistic confusion between "square" and "squared" which employs minimal coding to represent an important difference. However this confusion is not helped by a system of notation which itself confuses units and quantities.

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Homochiral creationism

SIR—

Cried God, making Adam from clay
In a dexterous left-handed way,
"Bring me PVED

ΔE_{pv}

Or I can't get it done in a day!"

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1. Tranter, G.E. *Nature* **318**, 172–173 (1985).

Further anxieties about AIDS

AIDS has been a serious problem of public health since it was first recognized. The realization that it may affect the human nervous system gives a new twist. But the remedies are the same.

THE letter from the Drs Fox on page 8 has plainly been written to scare, but it should be taken seriously for all that. Since AIDS (acquired immune deficiency syndrome) was first recognized, it has been seen as a serious threat to public health. It is intractable and infectious, although not especially so. What the Drs Fox (respectively a pathologist and a physician) are saying is that the threat is probably more serious than has been thought, now that it has been demonstrated that infection by the AIDS virus, HTLV-III or LAV, can damage the central nervous system. But it is too soon to know how much more serious.

While so much remains to be understood about the consequences of infection by the AIDS virus, it is not surprising that uncertainty abounds. But the wonder is that so much has been learned about AIDS and its causes in a mere four years.

The immune deficiency that marks out AIDS arises because of the depletion of the sub-class of T (for thymus) lymphocytes known as "helper" cells. Infected people produce characteristic antibodies, by means of which diagnostic tests have been developed and put into service. Their most immediate use is in the screening of blood for transfusions; it is too early to know how effective they will be.

Two lessons have emerged from the determination in the past year of the nucleotide sequence of several isolates of the virus. Viruses recovered from different sources differ — suggesting antigenic variability like that which makes the influenza virus variable — and the AIDS virus is less like HTLV-I (recovered from patients with apparently infectious adult T-cell leukaemia) than visna virus, responsible for a progressive (demyelinating) disease of the nervous system of sheep.

A great deal has also been learned about the nature of AIDS itself. That only some, perhaps 10 per cent or so, of those with HTLV-III antibodies succumb to one of the infections or other conditions that characterize the disease is not surprising. Other viruses may affect people subclinically, causing them little trouble but stimulating the production of antibodies which protect against further infection.

Much has also been learned about the mechanism of the spread of AIDS. Homosexuals, haemophiliacs given contaminated blood-clotting factor and syringe-using drug addicts are the groups chiefly at risk, but the disease has also

been transmitted to heterosexual partners of homosexual men and by infected women to their children *in utero*. Blood-to-blood transmission is the surest route to infection. Little is known about the effectiveness of other methods of transmission, but the AIDS virus is plainly not particularly infectious. Even so, it is reasonable to expect that in due course the infection will spread through the general population in the narrow sense that the groups now most at risk will be less conspicuous. This is what seems to have happened in some parts of Central Africa.

It is a triumph that questions like these have been successfully answered in so short a time. Even so, there remain some curious puzzles. If, for example, the underlying defect in AIDS patients is immunosuppression, why should the rare skin cancer called Kaposi's sarcoma, believed by some to involve cytomegalovirus as a kind of co-carcinogen, be such a common sequel to infection by HTLV-III? Why not other kinds of cancer? And what is the status of those who carry antibodies against HTLV-III but show no overt AIDS? Are they patients in waiting, just lucky or is there an explanation of their apparent immunity?

This is the point from which the Drs Fox start. What, they ask, if those without overt AIDS who have been infected by the virus are at risk of succumbing instead to one of the neurological conditions associated with overt AIDS? What would happen if psychiatric hospitals were refilled with relatively young people suffering from conditions at present associated with old age, dementia for example? Could we shoulder the social burden? The question is chilling. Is it fair?

Over the past few months, there has been a trickle of articles in the clinical journals pointing to a neurological component of AIDS. From the outset, physicians have recognized what is vaguely called "AIDS-related encephalopathy" in some overt infections. The new development (see, for example, Ho *et al.* and Resnick *et al.*, both in *New. Engl. J. Med.*, 313, 1493 & 1498; 1985) is the recognition that HTLV-III virus may infect brain tissue (promoting the formation of antibodies in the cerebrospinal fluid) and that it may be directly responsible for some of the damage done to the nervous systems of people with AIDS. More ominously, some of those in whose nervous tissue HTLV-III has been found are people

without overt AIDS.

These investigations are still few and far between. Knowledge of the neurological complications of AIDS itself is only sketchy. As yet, there is no factual basis for an attempt to guess at the frequency with which virus reaches the brains of those who have merely been infected with the virus. But if the apparent similarity between HTLV-III and visna virus is sustained by further investigations, and because HTLV-III is a retrovirus that may become a part of the genome of somatic cells and nervous tissue in particular, there is a danger that AIDS infection may be hidden in the brains of human beings much as the herpes simplex virus (which causes "cold sores") may linger dormant in the peripheral nervous system, bursting out only from time to time.

This is the background against which the Drs Fox have written their scary letter. There is a possibility that HTLV-III will lodge in infected people's brains, serving both as a cause of direct neurological damage and as a reservoir for the infection of others. Plainly there is an urgent need that this possibility should be explored, not least because virus in the brain may be less accessible to therapeutic drugs. Plainly, that will take time.

What, in the meantime, should be done? Reactions to the emergence of AIDS as a public health problem in industrialized communities has spanned the usual spectrum from panic to complacency. Many have responded with exaggerated fear to the notion that casual contact may infect them, while the primitive response of those who believe that Lot's wife met her deserved fate on the escape from Sodom and Gomorrah has been distasteful and unhelpful.

A strategy for containing AIDS can consist only of two components — some means of decelerating the spread of infection (which is a short-term measure) and, later, some means of preventing infection of those at risk. Infection by contaminated blood products should soon be halted. No doubt the fear of infection has already had an important influence on the behaviour of homosexuals, perhaps even drug addicts. But the benefits will emerge only slowly and the prospects for prophylaxis and cure are still distant. The recognition that there are probably neurological consequences of infection by HTLV-III makes a serious problem of public health still worse.

John Maddox

Immunology

Approaches to AIDS therapy

from David Klatzmann and Luc Montagnier

MUCH publicity has been given to the announcement by a group of French physicians of their treatment of acquired immune deficiency syndrome (AIDS) with cyclosporin A, an immunosuppressant. After four days of treatment with the drug, the T4 lymphocyte count of one patient rose from 4 to 250 cells per microlitre (900 ± 400 being the normal value), most of which seemed to be immature lymphocytes. But it was later announced that whereas 10 patients with AIDS or the AIDS-related complex had shown similar initial improvements in T-lymphocyte counts, the original patient had died 15 days after treatment began. At first sight, it may well seem counterproductive to treat an immune deficiency disease with an immunosuppressant. There is some theoretical basis^{2,4} for such treatment if AIDS is considered to be a virus-induced autoimmune disease but, because of the hazards of immunosuppressive treatment, the evidence for and against AIDS as an autoimmune disease needs to be carefully considered.

The most frequent clinical expression of AIDS is the occurrence of severe opportunistic infections, reflecting a profound defect of cellular immunity. Laboratory analysis demonstrates a broad spectrum of immune abnormalities⁵, among which is a drastic reduction in the numbers of T4 lymphocytes in peripheral blood and secondary lymphoid organs as well as an impairment of their functions^{6,7}. This may be accounted for by the biological properties of the AIDS virus, LAV² (also known as HTLV-III or ARV) which displays a selective tropism for normal T4 lymphocytes both *in vitro* and *in vivo*⁸. Intensive replication of the virus in cultured T4 lymphocytes is accompanied by a cytopathic effect which includes inhibition of cell growth, virus-induced fusion of the plasma membrane and, eventually, destruction of the cells⁹.

It is unlikely, however, that AIDS is the result of a direct progressive destruction of T4 cells by the virus for at least two reasons. The first is that the replication and cytopathic effect of LAV can only be observed in activated T4 cells^{2,9}. Indeed, LAV infection of resting T4 cells does not lead to viral replication or to expression of viral antigen on the cell surface, while stimulation by lectins or antigens of the same cells results in the production of viral particles, antigenic expression and the cytopathic effect. An equivalent phenomenon has been extensively documented for visna virus¹⁰, the expression of which is associated with the activation

of latently infected macrophages¹¹. Visna virus is the prototype of the lentivirus family of retroviruses and comparison of LAV and visna virus nucleotide sequences strongly suggests that LAV is also a lentivirus. Indeed, comparison of protein and nucleotide sequences of various retroviruses generates phylogenetic trees demonstrating that not only LAV and visna but also caprine arthritis-encephalitis virus and equine infectious anaemia virus (the latter being antigenically related to LAV¹²) are members of a subfamily of retrovirus that diverged early from other retroviruses such as oncoviruses^{3,13}.

The second reason to doubt that the destruction of T4 cells by LAV is the cause of AIDS is that only a small proportion of these cells is infected by the virus. It is not clear why this is so because, by definition, all T4 cells carry T4 molecules on their surface and these molecules seem to act as the receptor by which LAV enters the cells^{14,15}. And yet in purified T4-cell cultures, even at the peak of virus replication only 5–10 per cent of the cells express viral antigen and are subsequently lysed by the cytopathic effect⁹. Furthermore, only 10–20 per cent of clones derived from the CEM T4 cell line are susceptible to LAV infection, even though they all express the T4 molecule on their surface¹⁶. A similar picture emerges from studying patients: *in situ* hybridization with viral DNA probes indicates that less than 1 in 10,000 lymphocytes are replicating LAV at a given time¹⁷ and the Southern blot hybridization technique, which can detect as little as one copy of viral DNA per cell, yields negative results on fresh peripheral blood lymphocytes of AIDS patients¹⁸.

Taken together, these observations provide evidence against direct destruction of the T4-cell system by virus infection and replication. Therefore, one has to envisage alternative theories that explain the impairment in T4-cell number and function that characterizes AIDS. What could these be?

Immunosuppressive proteins

One possibility could be that LAV, like human T-cell leukaemia viruses I and II¹⁹ as well as mouse and cat leukaemia viruses, possesses a protein in its envelope (p15E) that is able to induce immune depression of T-cell function. But no such protein seems to be produced by LAV. Various reports have suggested that sera of AIDS patients contain soluble factors that can suppress *in vitro* T-cell functions²⁰. Such factors have not been characterized and their viral origin has not been proven but at least the obser-

vation that viral proteins are released during *in vitro* LAV production by lymphocytes²¹ is consistent with the idea. The availability through genetic engineering of viral purified proteins should help in testing this hypothesis in the near future.

Autoimmune mechanisms

The role of viral infections in triggering autoimmune diseases is well documented. For instance, diabetes in mice can be caused by an immune destruction of Langerhans cells following their infection by viruses such as coxsackie strains²². In retroviral infections, such a mechanism has been invoked for the encephalitis induced by visna virus and the haemolysis induced by equine infectious anaemia virus. AIDS patients often display signs of autoimmune reactions that are probably secondary to cell destruction caused by various opportunistic infections, but a more specific autoimmune process may be triggered by the ability of LAV envelope proteins to bind to T4 molecules. Indeed, not only virions but also free LAV proteins released from LAV-producing lymphocytes may bind to T4 molecules on the surface of uninfected lymphocytes. This could explain the general disappearance of detectable T4 molecules on the surface of LAV-infected cultured T4 lymphocytes in which only 10 per cent of cells seem to express viral antigens.

Whether this also occurs *in vivo* is not known, but if it does, the result could be that non-infected lymphocytes become highly immunogenic because of their surface-bound T4 molecules. This could account for a general destruction of the T4-cell system by classical cellular and antibody-mediated cytotoxicity⁴. The whole pathophysiology of AIDS would then be the result of a situation in which the few LAV-infected cells in the resting state are invisible to the immune system because they do not express viral antigens. Each time these cells are stimulated, not only they but also many non-infected cells will be destroyed by the immune system because they have become coated by LAV proteins. Thus, the immune deficiency induced by LAV infection would be the result of an active autoimmune destruction of the T4 lymphocytes, probably enhanced by the repeated stimulation of the immune system by antigens or alloantigens.

Rational therapy

According to the pathophysiology of the disease, three therapeutic approaches to AIDS can be distinguished. The first is the development of drugs to destroy viral particles and infected cells, or at least stop the virus spreading. After *in vitro* screening, drugs such as HPA23, suramin and ribavirin have demonstrated antiviral activity and are now being tested in controlled clinical trials. Preliminary results indicate that LAV can-

not be isolated from patients' lymphocytes during treatment but that the virus reappears at the end of treatment; moreover, the control of virus spreading is not associated with an immune or clinical improvement^{23,24}.

A second approach involves immune stimulation or reconstruction. Immuno-stimulators alone, even pure interleukin-2, seem to fail to reconstitute the immune system of AIDS patients. Interferon, which has both antiviral and immuno-stimulant effects, seems to be of value in the treatment only of Kaposi's sarcoma, which is often associated with AIDS. In any case, since cell activation probably contributes to virus spreading and T4 depletion, the use of such drugs in patients with minor symptoms of LAV infection may be dangerous in that it could accelerate progression of the disease unless antiviral drugs were used at the same time. Immune reconstitution by bone-marrow graft or leukocyte transfusion has been found not to work because the grafted cells are rapidly reinfected by the virus²⁵.

The third approach would be based on the view that AIDS is an autoimmune disease. This would suggest that immunosuppressive drugs, which can limit both cell stimulation and cytotoxic mechanisms, have a role in the treatment of AIDS. In that case, treatment would be designed first to control virus replications and then to 'tolerate' non-infected cells coated with viral proteins, in the way an allograft can be tolerated. Since there is still no firm basis for such a therapeutic approach, which is potentially dangerous, caution is required before clinical trials. While immunosuppressive treatment has been used in visna-infected animals with clinical improvement of early brain lesions without any adverse results²⁶, there are descriptions in the literature of patients who have

died of fulminant *Pneumocystis carinii* pneumonia after being treated with immunosuppressive drugs in ignorance that they were LAV-infected²⁷. Even if cyclosporin A has some potential use in virus-induced autoimmune disease, its use in AIDS patients should await more experimental knowledge about its action on T cells, which may be highly complex²⁸.

Finally, because of the complexity of

the pathophysiology of AIDS, only active and constant cooperation between biologists and clinicians will lead to an effective therapy, probably involving a combination of drugs and other treatments. □

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Weather forecasting

Storm hunting with fractals

from A. Hollingsworth

MRS BEETON might well have observed that to make tiger soup, first catch your tiger. Tigers for a weather forecaster are vigorous storms that put life or property at risk. They occur on scales ranging from the tornado (~1 km) to mid-latitude cyclones (~2,500 km). Locating the whereabouts of a storm and estimating its ferocity are the first steps in making a useful forecast, but the surface meteorological network is very inhomogeneous, with holes of many sizes where storms can lurk undetected.

On page 43 of this issue, Lovejoy *et al.* describe this problem in the language of fractal analysis. They give a definition of the dimension of an observing network, and argue that if the dimension of the network is lower than the dimension of the field being observed, many of the most energetic entities will never be detected. Many geophysical networks including the surface weather network are patchy in coverage and therefore lack dimensional resolution. Lovejoy *et al.* argue that long-term averages of geophysical fields derived from such dimensionally incomplete networks may be substantially biased: because of the inhomogeneity of the surface network, rare but vigorous events can be missed.

This suggests a bleak view of the feasibility of useful weather forecasting, a view which is belied by the fact that economically useful forecasts are routinely made out to perhaps 3 days in the tropics, 4 or 5 days in the southern hemisphere, and 6 or 7 days in the northern hemisphere extratropics. The success of the forecasts depends heavily on data sources other than the surface network as well as on sophisticated data-assimilation systems; ultimately success depends on the fact that scale separation is a meaningful concept for many practical purposes.

Data from polar orbiting and geostationary satellites, from the balloon network and from ships and aircraft provide the main basis for routine forecasting. The geostationary satellites, of which four are currently operational, provide multi-channel measurements every half-hour

over their field of view, which is roughly within 55° of the nadir point at the equator (Fig. 1). Cloud track winds can be derived from successive half-hourly scans in the visible and infrared. The satellites have a resolution of 1 km at nadir, and are invaluable for surveillance of tropical disturbances on all important scales. Two polar orbiting satellites provide radiometric soundings with good horizontal resolution four times a day, for most parts of the globe (Fig. 2). Vertical structure in humidity and temperature can be derived from the soundings; in middle and high lati-

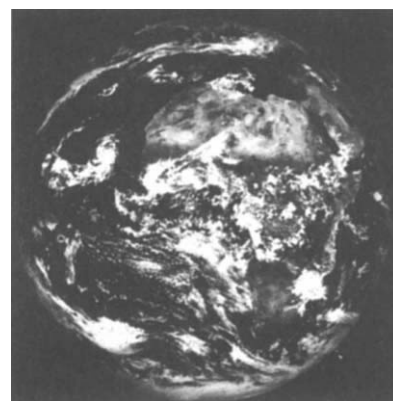


Fig. 1 Meteosat picture for 12.00 on 17 September 1985. The disturbance off the West African coast is the African wave that eventually developed into Hurricane Gloria. Cloud track winds can be estimated from successive half-hourly scans. The infrared channels enable one to estimate the height of the clouds. Pixel resolution at nadir corresponds to 1 km.

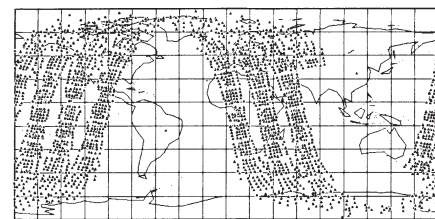


Fig. 2 Data coverage from a polar orbiter in a single six-hour period, covering about three orbits. A single orbiter can monitor every point on Earth twice a day.

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tudes, the temperature soundings are valuable in inferring the winds.

The data from these diverse sources are integrated in a coherent view of atmospheric structure in space and time using four-dimensional data-assimilation methods. At the core of these techniques is a sophisticated global forecast model which provides rather accurate estimates of the expected state of the atmosphere, based on earlier observations. At any given time the observations are insufficient to determine the atmospheric structure.

Information from the forecast model is used in conjunction with the latest observations in a Bayesian estimation of the true state of the atmosphere; the forecast model is essential to resolve the underdeterminacy of the observations. There are many analogies between the methods used in this field and those used, for example, in computational vision (see Poggio, T., Torre, V. & Koch, C. *Nature*

317, 314; 1985) or the applications of the Kalman-Bucy filter in control theory (see *Data Assimilation Methods* eds Bengtsson, L., Ghil, M. & Kallen, E., 330; Springer, 1981).

Global weather forecast models solve a version of the Navier-Stokes equations for fluid flow on a grid which has a horizontal resolution of about 150 km and a vertical resolution of 1–2 km. Some important weather cannot be resolved on such a grid. The fact that one can nevertheless make useful forecasts suggests that the simplification of treating the atmosphere as smoothly varying on the smallest resolved scales is not a serious error. Statistical methods can, if necessary, be used to interpret the forecast in such a way as to recover useful local detail. □

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Immunoglobulin genes

Inversion for gene construction

from David Baltimore

JOINING of immunoglobulin genes as an event of immunodifferentiation is not unique in the biological world. Recombination of DNA molecules is commonplace and serves many functions, including meiotic recombination, post-replicative repair, phase variation in *Salmonella* bacteria and the generation of transcribable surface antigen genes in trypanosomes. Although DNA recombination frequently occurs, somatic rearrangement of gene fragments to produce functional genes is at present known to be used only by the immune system. This specificity implies that the rearrangement process should be active only in particular cells and be targeted only to well-defined DNA segments. On page 28 of this issue Malissen *et al.*¹ have extended our understanding of the rearrangement process by their analysis of the T-cell receptor gene complex.

The nature of the enzymes involved in the immunoglobulin gene rearrangement process, their control and their targeting specificity remain mysterious — we are still trying to understand the ground rules. One of the more obscure aspects is the relative placement and orientation of the gene fragments to be recombined to produce the immunoglobulin molecule. Although textbooks and the diagrammatic slides shown by many immunologists blithely display strings of variable (*V*), diversity (*D*) and joining (*J*) segments, the only situation where a complete physical linkage has been achieved for all the elements of an immunoglobulin gene-segment joining system is with chicken lambda genes². For all mammalian sys-

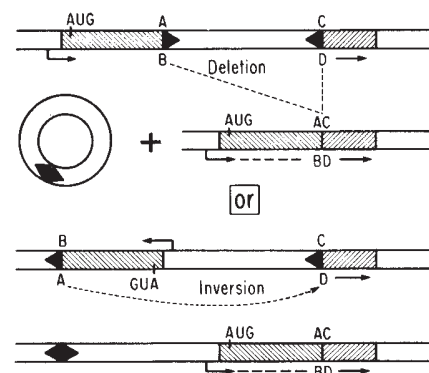
tems, the relative positions and orientations of the elements to be joined are incompletely established. Various arguments give a strong indication of the organization and orientation of the immunoglobulin heavy-chain gene segments^{3,4}, but the situation for the kappa light-chain genes is uncertain. The joining of kappa-chain gene segments involves a recombination between one of many hundreds of *V* segments with one of four *J* segments.

Although the *V* and *J* segments have not been physically linked in recombinant DNA clones, Lewis *et al.* in 1982 proposed that at least some of the kappa *V* regions occur in the DNA chain in the opposite transcriptional orientation to the *J* segments⁵. This proposal was based on the observation that in certain cells that have productively rearranged their DNA, segments of DNA remain that should have been deleted if both *V* and *J* were found in the same orientation^{5–7}. If a *V* segment were in the opposite orientation to a *J* segment, joining the two elements would have to invert the intervening DNA rather than deleting it. Such an inversion would not remove any large segments of DNA from the cell but would simply reconfigure the existing DNA. This proposal met with much disbelief largely, I believe, because the DNA gymnastics involved seemed formidable although, in fact, they are quite ordinary (see the figure).

A decision about whether inversional joining is a mechanism of rearrangement was approached in two further papers by Lewis *et al.*, who used an artificial DNA construct with inverted *V* and *J* segments

that would rearrange after integration into certain cells^{8,9}. This work showed that inversional joining is a feasible mechanism, but left open the question of whether it actually occurs in normal B-cell maturation. Two indirect pieces of data argued for inversional joining^{10,11}, but both involved abnormal events. The direct demonstration of inversional joining in the kappa gene of a plasmacytoma was made by Feddersen and Van Ness¹², who found that they could recover both products of a natural joint and showed that the two had to arise by inversional joining. This directly implied that some *V* regions are in opposite orientation to the *J* regions.

Malissen *et al.* have now extended the analysis to the *V* regions of the T-cell receptor gene complex. Here they have uncovered a most surprising situation (one that could as easily have been true of kappa light-chain genes and may yet turn out to be). They have shown that at least one *V* segment is downstream of the constant (*C*) regions. In all previously characterized systems of immunoglobulin-related genes, the *V* segments have been thought to be upstream of the other segments. For immunoglobulin heavy-chain genes, joining is accompanied by deletion of the intervening DNA¹ and this requires that the



The difference between deletional and inversional joining. The difference is solely in topological orientation of the two participating pieces of DNA. The units to be joined (the *V* and *J* units shown here) are identical but in one case they are located in the DNA in the same transcriptional orientation and in the other in opposite orientation. The biochemistry of joining is identical in both: the same sequences are joined to one another. This can be seen by following the locations of the hypothetical sequences A, B, C and D. In both cases of joining, A is joined to C and B is joined to D. The consequences of deletional and inversional joining are quite different. In the first, a segment of DNA containing the joining signals is released as a circle and discarded from the cell. In the second, no segment of DNA is lost from the cell but the inversion causes the back-to-back apposition of the joining signals. ↳, Site of initiation of transcription with direction indicated; AUG, codon in *V* region that initiates translation; ►, signal encoded in the DNA that specifies a site for joining (a conserved heptamer and nonamer).

V regions be upstream of the C regions (if they were downstream, the C region itself would become part of the deleted DNA).

For a V region to be downstream of the C region, topological considerations dictate that the V region must be in opposite transcriptional orientation to the C region. Malissen *et al.* were able to verify this because the V and C regions are near enough to each other to be physically linked in cloned DNA. The structure that they uncovered, in fact, implies that the joined segment in the T-cell clone arose by an initial deletional join of an upstream D segment to a J segment followed by a V-to-D-J inversional join in the downstream direction.

It should be emphasized that all of the DNA mechanics that have been found to underlie T-cell receptor gene rearrangement are congruent to the events of immunoglobulin gene rearrangement. The DNA signals thought to direct the rearrangement process are indistinguishable in the two systems. Malissen *et al.* reinforce this perspective by showing that D-to-J rearrangement precedes V-to-D-J rearrangement as found for immunoglobulin heavy chains. They also note that the joined genes have lost potential coding nucleotides found near the joining signals in the germline DNA elements and that new nucleotides (constituting an N region) are found between the joined V and D segments. The only surprise is that the reciprocal joint formed during the re-

combination retains three nucleotides between the heptamers. In all cases of immunoglobulin gene joining yet studied, the heptamers are joined back-to-back with no intervening nucleotides (in one case a join with one nucleotide missing was found; ref. 9).

With this demonstration of inversional joining of a T-cell receptor V gene segment, most of what we want to know about the topology of joining is available. All we now need is to understand the sequence requirements for joining; then we can turn to enzymology, a task already begun in many laboratories but with little reported success. □

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Plate tectonics

Global thermal histories

from Mike Bickle

TECTONIC geology is a manifestation of the thermal evolution of the interior of the Earth. Surprisingly, models for this thermal evolution have proved difficult to reconcile with a geological record that extends over three-quarters of the history of the Earth. The relatively low crustal thermal gradients recorded in the earliest rocks are incompatible with the higher gradients predicted by convection models that incorporate the higher radiogenic heat production that must have then prevailed. After re-examining this problem, F.M. Richter (*Earth planet. Sci. Lett.* **73**, 350; 1985) concludes that low continental thermal gradients could only have been maintained in the Archaean if the continental lithosphere was stabilized by chemical means (that is, was less dense), rather than being a simple thermal boundary layer as seems appropriate for much of the present-day lithosphere. The concept of a chemically stabilized lithosphere is not new (see, for example Jordan, T.H. *Nature* **274**, 544; 1978) but it now seems that such stabilization was of greater importance in the

past and may have had a profound effect on the evolution and preservation of ancient continental crust.

Models for global thermal evolution allow calculation of the temperature dependence of heat loss for a spherical, layered and convecting Earth. To be successful, a model must not only yield a plausible range of present-day interior temperatures (given an appropriate initial state), but must also account for the higher radiogenic heat production and loss of initial heat formation that must have occurred in the past. Successful models achieve this by generating internal temperatures higher than at present and thinner boundary layers with steeper thermal gradients.

Good evidence for higher internal temperatures is provided by komatiitic lavas, which are restricted to the Archaean and were erupted at temperatures about 200°C higher than any recent lavas. However, the geological evidence from metamorphic rocks, crustal thickness and sedimentary basin subsidence in-

100 years ago

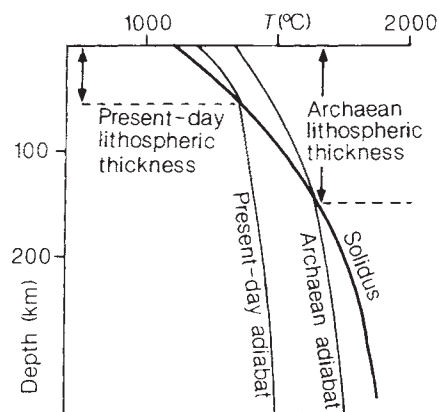
PHYSICS AT JOHNS HOPKINS

The large and well appointed laboratories recently erected by the Trustees of the Johns Hopkins University for the Chemical and Biological Departments have by contrast made the more evident the needs of the Physical Department, which has been obliged to occupy temporarily parts of four different buildings for a physical laboratory. The new laboratory is to be a handsome building of red brick, trimmed with brown sandstone, and will occupy a fine site about a block from the other University buildings, on the corner of a quiet little street midway between the more important streets, which carry the bulk of the traffic. It will therefore be as free from disturbance from the earth-vibrations as could be expected in a city. The building will be 115 feet long by 70 feet broad, and will have four stories besides the basement. In the centre of the building, and below the basement, are several vaults for instruments requiring to be used at constant temperature, also a fireproof vault for storage. In these vaults will be placed Prof. Rowland's dividing-engine, by which the diffraction-gratings are ruled, and the Rogers-Bond comparator, which has recently become the property of the University. There will be a tower on the south-east corner which will be provided with telescope and dome, and will be a convenient observatory when great steadiness in the instruments is not required.

from *Nature* **33** 236, 7 January 1886.

indicates that the range of crustal thermal gradients in the Archaean may not have differed significantly from the range of modern continental thermal gradients. This inference is strongly supported by the demonstration of Archaean ages for diamonds (Richardson, S.H. *et al.* *Nature* **310**, 198; 1984). The diamonds must have been preserved since roughly 3,200 Myr ago at depths greater than 150 km in continental lithosphere of at least similar thickness to present day continental lithosphere.

The problem addressed by Richter is how to allow higher interior temperatures without affecting that part of the boundary layer under the continents. Previous solutions have noted that heat is lost from the Earth's interior by two mechanisms: an unsteady small-scale convection of sinking and rising jets transporting heat to the base of the lithosphere through which it is subsequently lost by conduction; and larger scale recirculation of the whole lithosphere in oceanic regions, that is, plate tectonics. The driving and resisting forces involved in the second mechanism are difficult to quantify and there has been a temptation to assume that plate tectonics can transport any necessary extra heat. Richter has developed regional models to try to account for heat lost by both mechanisms as a function of internal temperatures. He looks particularly at the interdependence of the mechanisms. All his models with thermal boundary-layer definitions for the lithosphere result in boundary-layer thermal gradients that in-



Depth-temperature plot for the upper 200 km of the Earth, showing the intersection of the present-day and supposed Archaean temperature profiles with the mantle solidus (the locus of the onset of melting). Because of the increased depth of initiation of melting in the Archaean, the depleted lithosphere would have been thicker than today.

crease at a rate faster than the increase in heat production. This is because the effectiveness of plate-tectonic heat loss decreases as temperatures increase and plate thicknesses decrease. The only viable resolution of this problem is that the continental lithosphere is stabilized chemically rather than thermally.

How robust is this conclusion? All convection calculations involve a series of assumptions. U.R. Christensen (*J. geophys. Res.* **90**, 2995; 1985) has performed numerical calculations of convection in a fluid with a temperature-sensitive viscosity and raised some important questions about the validity of the parameterized calculations used by Richter. Such calculations estimate the temperature dependence of heat loss by comparing numerical convection models run at various newtonian viscosities, but with constant viscosity in each model. Christensen's experiments with viscosity as a function of temperature indicate that heat flux is less sensitive to temperature than previously thought. This is because the cooler higher-viscosity upper boundary layer controls convection rates; hence the heat flux is related to some temperature in the upper boundary layer rather than the substantially higher interior temperature used as a scaling factor in the parameterized calculations. Christensen's calculations indicate how much has to be learnt about convection in variable viscosity and non-newtonian fluids appropriate to the Earth's mantle.

Nevertheless, such complexities seem unlikely to affect Richter's basic conclusions. Heat flux from the Earth is necessarily fixed at a rate sufficient to allow the Earth to cool to its present state from initial temperatures high enough to allow substantial but incomplete outgassing. In addition, Richter's calculations span a range of viscosity-temperature relationships including the limiting case of viscosity independent of temperature.

Interior temperatures are less well constrained by parameterized models, but no model based on the lithosphere as a simple thermal boundary layer allows global heat to rise without increased heat input into the continental lithosphere.

The heat loss considerations allow some interesting speculations about Archaean tectonics, which are important because they lead to possible tests of the hypothesis of a chemically depleted continental lithosphere. Increased mantle temperatures cause melting to be initiated at greater depths. For example, at 1,600°C, appropriate to the Archaean mantle, melting might be initiated at about 150 km under mid-ocean ridges compared with depths of roughly 60 km for modern mantle (at approximately 1,350°C). This could yield a depleted lithosphere 150 km thick with 25 km of oceanic crust (see figure). The former might be converted to continental lithosphere by accretion of island arcs. Stabilization of the lithosphere by depletion to the depth at which melting is initiated seems essential to preserve Archaean crust from an alternative tectonic mode postulated by Richter. If convection penetrated into regions of partial melt below continental lithosphere the overlying crust would be continuously buried beneath

copious volcanicity. This would lead to subsidence, stabilization of a cool dynamically thickened thermal boundary layer and continuous recycling of the volcanic material down into the underlying mantle. Such a process is incompatible with preservation of Archaean crust and if it operated must have been restricted to a pre-Archaean period or perhaps to those areas of Archaean crust not preserved.

The postulate of a thick chemically depleted continental lithosphere is testable. Isotope data, including those yielding the age of garnet inclusions in diamond, can demonstrate the antiquity of peridotite xenoliths (such as are found in kimberlite pipes) putatively derived from the continental lithosphere. Their chemistry can indicate the degree of chemical depletion. A critical point yet to be resolved is whether the density decrease due to chemical changes is adequate to stabilize the lithosphere against the thermally induced density increase which limits the thickness of modern oceanic lithosphere (Houseman, G. & McKenzie, D.P. *Geophys. J. R. astr. Soc.* **68**, 133; 1982).

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Protein structure

Polar pocket with nonpolar lining

from David J. Cowley

CLASSICAL crystallographic studies of the globular protein myoglobin (Kendrew, J.C. *et al.* *Nature* **185**, 422; 1960) showed the oxygen-carrying plate-like haem group to be embedded in a pocket that is lined largely with nonpolar amino acids. But on page 70 of this issue R.B. MacGregor and G. Weber report optical spectroscopy studies with a new fluorescent probe, which indicate that the haem cleft has a polar interior.

Like the commonly used probe aminonaphthalene sulphonate, the new probe DANCA* has spectroscopic properties that reflect the polarity of its environment. But it has the advantage that the dipole moment on electronic excitation changes markedly in strength but not in direction. Such changes, detected spectroscopically and compared with the probe's optical properties in a series of homogeneous solutions of defined polarity, allow the ready estimation of the local polarity of a biological macromolecule enveloping the rigid probe chromophore.

DANCA has a high affinity (dissociation constant 1.2×10^{-5} M at 24°C) for apomyoglobin because its carboxylic

acid group mimics that of haem itself. PRODAN*, which has neither the high affinity for apomyoglobin nor the carboxylate group of DANCA, nevertheless has nearly identical absorption and fluorescent properties and can therefore be used for calibration purposes for solvents in which DANCA is insoluble.

Bound DANCA can be displaced stoichiometrically from apomyoglobin by one equivalent of haemin, showing that the probe is not loosely associated at a variety of sites but is located only in the protein pocket. On binding of DANCA to apomyoglobin in aqueous buffer at pH 6.0, the fluorescent emission intensity increases fourfold and the emission band shifts to higher energy. The emission spectrum is similar to that of DANCA in the very highly polar solvent *NN*-dimethylformamide at 20°C or in glycerol at -40°C. It is the gross redshift of the emission band relative to PRODAN in nonpolar cyclohexane solvent that reveals the high effective polarity of the interior of the myoglobin pocket.

The apparent paradox of a pocket lined with nonpolar amino acids and yet with a high effective polarity is resolved if the amide dipoles (C=O...H-N) disposed around the rim of the segments of alpha

*DANCA is 2'-(*N,N*-dimethylamino)-6'-naphthoyl-4-*trans*-cyclohexanoic acid; PRODAN is 6-propionyl 2-(*N,N*-dimethylamino)naphthalene.

helix forming the haem pocket can exert substantial electric fields inside the pocket despite the attenuation by the insulating lining of hydrocarbon side chains (see figure). Calculations confirm the feasibility of this explanation, which may apply to many enzyme interiors.

To infer environmental polarity from fluorescence band positions requires that the surrounding medium has had the opportunity to undergo a full adjustment to the excited chromophore before the latter emits light. Otherwise the emission spectrum shifts to longer wavelengths as longer wavelengths of light are used to excite selectively more stabilized solvated chromophores. This 'red edge effect' is observed for PRODAN in glycerol at -42°C but is absent above 1°C . Since 'solvent' relaxation must be complete, dependence of the emission of DANCA-myoglobin on the wavelength of excitation at room temperature is attributed to a heterogeneity of probe orientations within the haem pocket. Haem itself binds in two distinct orientations. The reported steady-state fluorescence studies provide limited information. Time-resolved spectra and fluorescence anisotropy decay data would enable the rotational constraints on the bound probe and the exchange rates between probe configurations to be evaluated.

An additional redshift of fluorescence, above the expected solvent relaxation shift, incipient at -35°C and almost fully developed at 4°C , is intriguing. Despite the claimed merits of DANCA as a probe of well-defined geometry and electronic nature, the initial excited state may transform to a more polar, lower-energy excited state by twisting about the di-

methylamino-naphthalene bond, with a likelihood that depends on the polarity and viscosity of the constraining medium. Such processes have been documented for many intramolecular charge-transfer systems analogous to DANCA, the classic example being *p*-*N,N*-dimethylamino-benzonitrile (Kosower, E.M. *Acc. Chem. Res.* **15**, 259; 1982 and Grabowski, Z.R. *et al. Nouv. J. Chim.* **3**, 443; 1979). If this were to be the case the 'environment polarity' would be overestimated. Notwithstanding, the high polarity of the myoglobin haem pocket is real.

MacGregor and Weber calculate electrostatic interaction energies of a monopole charge within a square grid of locations in the haem plane, together with the partial charges of nitrogen and oxygen centres of the peptide backbone in the known crystallographic positions of deoxymyoglobin, on the assumption that the intervening nonpolar sidechains form

a dielectric of relative permittivity 3. Several orientations of the probe molecule, represented by a pair of opposite charges 0.84 nm apart on the grid, were found to be capable of producing spectral shifts in excess of the observed shifts of $\sim 1,000\text{ cm}^{-1}$.

On the basis of the often minor changes in acid dissociation constants of protic groups in water and inside certain proteins, it has been deduced that many protein cavities have a high effective polarity despite the lining of 'nonpolar amino-acid residues' (Warshel, A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4785; 1984). These deductions are corroborated by the calculations and optical evidence of MacGregor and Weber. □

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Glynn Isaac 1937–1985

GLYNN ISAAC died in a hospital in Japan on 5 October after a brave fight against a vicious, and as yet unidentified, infectious illness. Although 'officially' an archaeologist, he drew no boundaries between disciplines in his pursuit of evidence about the origins and early stages of human behaviour. His field contributions are important enough in their own right, but Isaac will mainly be remembered for his eclectic use of evidence and for his passionate insistence that the hypothetico-deductive method can, and should, be applied to problems of prehistory.

Glynn Isaac's introduction to archaeology began as a schoolboy and continued as a student in Cape Town, and came from A.J. Goodwin and Monica Wilson. His mentors at Cambridge were Graham Clark and the late Charles McBurney. In 1961 he accepted a research post with Louis Leakey in Kenya which was the beginning of his professional association with African prehistory. Louis and Mary Leakey had investigated the site of Olorgesailie between 1942 and 1954 and, with typical generosity and perspicacity, Leakey gave Isaac the responsibility of further excavating and analysing the site. Isaac's monograph on Olorgesailie is a classic of its kind, and contains clear evidence of his sensitivity to the confounding problems that the geological context can bring to the interpretation of archaeological sites.

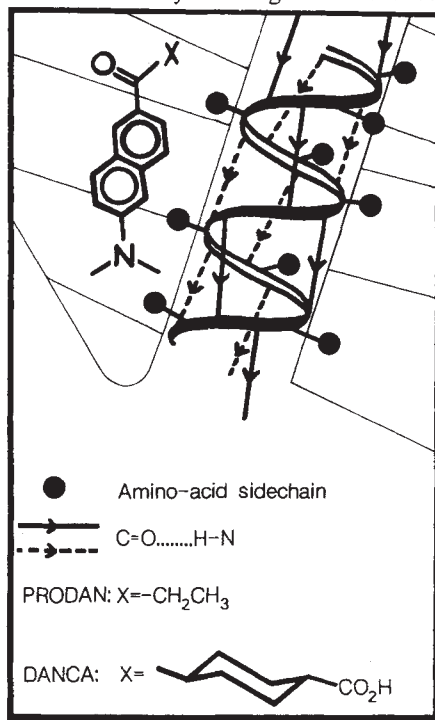
Field work in Nakuru and in the Natron Basin followed and it was at Natron, at Penini, that Isaac began his association with Richard Leakey. In 1969, Leakey invited Isaac to investigate the archaeological evidence which had then been recently discovered at the KBS site, at what was then East Rudolph, now Koobi Fora, in northern Kenya. However, Isaac's contribution to the

work at Koobi Fora was much wider, for he joined Leakey as co-director of the Koobi Fora research project and was instrumental in the development of nearly all aspects of the research that flowed from that area. His broad training in zoology and geology was crucial for this task. His strength as a scientist was most evident at the time when there was a conflict between the isotope and palaeontological evidence for the age of the KBS tuff. Isaac was determined to see, and was instrumental in achieving, the reassessment of the evidence. The apparent resolution of this problem proved to be the stimulus to important geochemical research which has resulted in more precise and accurate correlations of ash layers at four of the major East African sites.

Isaac was an inveterate hypothesis maker. His hypotheses about early hominid land use, behaviour and subsistence were mostly based on evidence that he and his collaborators had painstakingly recovered at Koobi Fora and elsewhere. The diverse and pertinent projects undertaken by his students, from studies of food availability to contemporary hunter-gatherers to the factors which disturb contemporary archaeological sites, speak for his willingness to put his own ideas to the test.

Isaac loved Africa and its people and he, more than any other, saw the need and the obligation to involve Africans in prehistory research. Prehistory is a field not usually noted for the humility of its practitioners but Isaac stood out as being wholly without self interest. His enthusiasms and influence spread far, and his impact on palaeontology, dating and on the conduct of anthropological field and laboratory research, as well as prehistoric archaeology, were more profound than I suspect we presently realize.

Bernard Wood



The haem cleft of apomyoglobin

Endangered species

The fate of the California condor

from David S. Wilcove and Robert M. May

CALIFORNIA condors (*Gymnogyps californianus*) have soared over the foothills and valleys of the western United States since the Pleistocene. Whether they will survive the twentieth century is another matter. Recent counts indicate that only six condors remain in the wild, with another 21 in captivity. The future of North America's largest bird rests with these 27 individuals.

The heyday of the condor is long past. Fossil remains indicate they once occurred from British Columbia in Canada to the southern extent of Baja California in Mexico, and eastwards across the southern United States to Florida. The rich Pleistocene mammal fauna as revealed in the Rancho LaBrea tar pits must have provided abundant food (carcass) and one can imagine the condors squabbling over the carcass of a mammoth. At that time, there were a number of giant scavenging birds in North America, of which the condor is the only surviving species.

Within historical memory, condors still occurred all along the Pacific coast of North America, yet almost as far back as the records go, they have been in decline. By 1850 they had almost disappeared north of California; by 1940 there were none left in Baja California. For most of the past 50 years, they have occupied a U-shaped area of about 45,000 km² from Santa Barbara south along the coastal range to San Jose and along the western foothills of the Sierra Nevadas to southern Madera county.

The first condor census, published in 1953 (ref. 1), gave an estimate of 60 birds, but this was obtained without marking individuals and is now believed to have been far too low². By 1978, only 25–35 condors were thought to remain in the wild. Four years later, some of the wild birds were brought into captivity and eggs were collected for captive hatching. The captive population has grown steadily but so far only by supplementary wild eggs and birds, because it will be several years before most of the captive birds are old enough to breed. The wild population, meanwhile, had dropped to 15 by October 1984 and another six birds had disappeared by May 1985, when three of the survivors were brought into captivity. All the wild birds today are radio-collared, which should allow biologists to monitor them closely.

Many explanations have been offered for the decline of the condor. In the vivid but teleological language of some earlier ecologists, it was termed a senile species: a bird whose low reproductive rate (one offspring every two years), lengthy adol-

escence (six or more years) and vast home range ill-suit it for life in modern-day southern California. The demise of the condor may, therefore, be simply a protracted coda to the larger extinctions that occurred about 10,000 years ago as recorded at Rancho LaBrea. Although there is some truth in this, for conservation purposes it is more useful to focus on the specific environmental problems that plague the condor, including molestation by humans, food shortages, environmental pollution and habitat destruction.

At the turn of the century, when a con-

IMAGE
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End of the line? (Frank Lane picture agency). Condor egg was worth as much as \$300, many of the nests were robbed. Federal protection has eliminated this threat but it has not prevented the occasional loss of adult birds to hunters. There is debate about the extent to which such vandalism is responsible for recent losses.

Some have suggested that the extinction of the Pleistocene megafauna resulted in a food shortage for the condor, but there were large herds of antelope and elk in the San Joaquin and Sacramento Valleys until the mid-nineteenth century. The expansion of ranching in this region provided the condors with abundant livestock, chiefly cattle, sheep and horses³, although the switch to agriculture in the early 1900s probably did reduce their food supply. A programme providing food supplements was started in 1971 (ref. 4).

A far more serious problem has been contamination of the food supply. Condors ingest lead when they dine on animals that have been shot by hunters⁵; at least two birds have died in the past three years from lead poisoning and lead levels in the remaining wild condors are alarmingly high. Levels of DDE in tissue samples

from wild condors are high enough to cause reproductive problems, assuming that condors are as sensitive as most birds of prey to DDE⁶. Eggshells of California condors from 1964–1969 were 33 per cent thinner than in the pre-DDT era⁶.

As the condors live in one of the nation's fastest growing areas, not surprisingly their habitat is under assault. Oil production and urbanization are continually extending into the grasslands where the birds forage, and mining and timber interests would like greater access to the mountainous regions where the birds roost and nest.

The US Department of the Interior must weigh all of these factors when deciding what to do with the remaining condors. Some scientists have recommended that the remaining wild birds be brought into captivity for safe-keeping. Their argument, which seems unanswerable to us, is that a programme of breeding captive birds is the only thing that can save the species, but others think that removing wild birds will eliminate any chances for re-introducing captive-bred individuals to the wild. Without older birds to show them where to roost, nest and forage, new recruits have little chance of surviving.

This argument epitomizes the choice that is likely to face us increasingly often, as more species are driven to the brink by human population expansion: do we settle for preserving specimens of each species in some form of zoo or do we strive, possibly hopelessly, to preserve the ecological entity with its full panoply of behaviour in the wild? In the case of the condor, some urge that a few be left wild, because it may be politically impossible to fend off the assaults on their habitat if there are no wild birds left⁷. The most recent agreement hammered out between the State of California and the Department of the Interior proposes the capture of three of the six remaining wild condors with simultaneous release of three captive birds, but the agreement is contingent on the department purchasing a 14,000-acre tract of land needed for condor conservation, and will probably collapse if any more wild birds die in the next four months.

California condors were around long before the first humans colonized the New World. It may be a measure of the success of our own species that we have all but eliminated the condor. Another measure will be whether we can now save it. □

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Trialkyl lead in rain in the Black Forest

SIR—We have recently suggested that high concentrations of trialkyl lead, R_3Pb^+ , as detected, for example, in rainwater samples from the Black Forest, may be harmful to the forests of Central Europe¹. The theory has been seriously questioned by M.H. Unsworth and R.M. Harrison². We wish to provide some new and supplementary data, which we believe can help to remove their doubts.

The most serious questions posed by the authors concerned the bioassay used for quantitation of R_3Pb^+ . In continuing our study for several months of this year on behalf of the government of Baden-Württemberg, two independent methods have been employed for the determination of R_3Pb^+ in rain water. In addition to the tubulin assay, all samples were submitted to a chemical separation procedure³ which was also used for the determination of some of the concentration values cited by Unsworth and Harrison. The results of both assays were the same within the limits of experimental error. Again, four of the samples collected at two locations in the Black Forest had trialkyl lead concentrations higher than 0.2 μM .

Unsworth and Harrison were concerned about the likelihood of the occurrence of such high concentrations of R_3Pb^+ in rain. The concentrations are indeed 100–1,000 times higher than those reported by others. So far, however, analytical data on trialkyl lead in rain are scarce. The few data known are from locations with a typical sea climate, which may not be comparable to locations on the Continent. Moreover, the occurrence of trialkyl-lead-polluted rain seems to be confined to small areas, as shown by the analytical results obtained from one of our sampling stations which, although located only 100 km east of the Feldberg, has never given R_3Pb^+ values above the limit of detection. Sampling at this site alone would have led to different conclusions. Finally, the Antwerp data mentioned by Unsworth and Harrison were one-sample measurements, and therefore statistically insignificant. In 1984, it took us 4 months of uninterrupted observation before we recorded a single toxic event.

Unsworth and Harrison argue that the low concentrations of R_3Pb^+ in the atmosphere cannot account for the high concentrations of R_3Pb^+ in rain. For the undergraded compound R_3Pb , Harrison and co-workers reported atmospheric concentrations at rural sites of up to 230 ng m^{-3} . After degradation, the distribution of R_3PbX can be expected to remain unchanged, at least for some period of time. However, values of R_3PbX were reported to be as low as 2 ng m^{-3} . So where is the rest? The loss is most easily explained by

the high instability of salts when dispersed in air. They may be captured by polar media, such as surfaces of particulate matter, and would therefore be analysed together with the inorganic lead. Indeed, in most of the studies of atmospheric lead pollution, the term "inorganic lead" stands for the lead fraction removed by filters. R_3PbX could be washed off the surface of such particles, on contact with rain or fog. In our example, 230 ng of R_3PbX would be dissolved in about 10 ml (10 g m^{-3} is the typical atmospheric humidity in Europe). The resulting concentration would be about $10^{-7} M$, which is within the range of R_3PbX found in the Black Forest. This value does not consider the R_3PbX that may be taken up by rain on its way from clouds to the surface.

Fortunately, we do not disagree on controversial theories but on a finding that can be examined experimentally. Therefore, we suggest that further discussion should be postponed until our results have been confirmed by others. Twin samples were taken of most of the rainwater samples collected in 1985 in the Black Forest. They are waiting to be analysed.

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An anniversary for the lipid bilayer

SIR—The year 1985 marked the sixtieth anniversary of the proposal by Gorter and Grendel¹ that biological membranes consist of a double layer of lipid molecules, as appropriately mentioned by Bretscher². In 1925 E. Gorter, a pediatrician with a strong interest in chemical pathology, and F. Grendel, a chemist in the laboratory of the University Children's Hospital at Leiden, The Netherlands, derived their cell membrane model from observations made with rather simple experimental techniques. The lipid extracted with ace-

tone from a known amount of erythrocytes was spread as a monolayer on a water surface. The ratio of monolayer lipid area to total erythrocyte surface turned out to be close to 2:1 for red blood cells from different mammalian species. Thus the investigators concluded with the suggestion that in the cell membrane of erythrocytes sufficient lipid is present for constituting a molecular bilayer. The structural model is the more remarkable because it was derived without any modern instrumental method such as infrared spectrometry, gas chromatography or electron spin resonance. Already 10 years ago, the Gorter-Grendel model was considered by Bretscher and Raff³ a major breakthrough in cell biology, comparable to that of the double DNA helix. In 1934 Danielli and Davson⁴ improved on the model by allowing for proteins to be additional components of plasma membranes. The fluid mosaic model proposed in 1972 by Singer and Nicolson⁵, viewing functional biological membranes as bidimensional solutions of integral proteins in a liquid phospholipid matrix, remains the current basis for experimental work.

In 1966, Bar *et al.*⁶ repeated the Gorter-Grendel experiments and discovered two major errors. First, acetone treatment extracts membrane lipids, and cholesterol in particular, incompletely. Second, more refined measurements of the surface area of the human erythrocyte yield the value of $145 \pm 8 \mu m^2$ instead of $99 \mu m^2$, the value used in 1925. The Dutch investigators had in fact made two mutually compensating errors of about 40%.

We are proud that in 1935 Dr E. Gorter, at that time chairman of the pediatrics department at Leiden, accepted an invitation from Ghent State University to come and found the first university children's hospital in Flanders, Belgium. The strong interaction between pediatric observation and biochemical research, kindled by Professor Gorter, and promoted continually by his pupil and successor, Professor Hooft, who died in 1980, remedied effectively the developmental delay of pediatrics in Belgium. We owe much to Evert Gorter, who was once called: 'The best chemist among all pediatricians and the best pediatrician among all chemists.'

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where the Matters Arising section remains appropriate).

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X chromosomes and dosage compensation

SIR—D.A. Smith (*Nature* 315, 103; 1985) uses the term "dosage compensation" to mean that effect on the rate of transcription of X-linked genes in the male which enables it to survive the lack of one X chromosome; X inactivation in the female is, then, a further compensatory step to enable it to get over the problem of over-active X-linked genes. By suggesting that evolution could have somehow "resolved" the situation arising from aneuploidy for an entire X chromosome in the mammalian male, G.P. Maroni (*Nature* 317, 22; 1985) asks whether the inferences drawn by Smith are valid. Purely in terms of the physiology of gene action, precisely what evolution had to "resolve" is not clear: in the course of their extensive analysis of the problem of dominance, Kacser and Burns¹ have shown that — special cases apart — metabolic fluxes are extremely insensitive to whether an enzyme is represented by one structural gene or two.

I wish to draw attention to a point that usually is not taken into account. Sex chromosomes are expected to include one or more sex-determining genes. If the mammalian X chromosome indeed contains genes determining primary sex, what such genes might require is not dosage compensation but full expression, or even exaggeration, of the difference in dosage between XX and XY genotypes. Thus it can be argued that dosage compensation (in the usual phenotypic sense) is unlikely to be the primary reason for X inactivation. Is it possible that X inactivation has a function more vital than dosage compensation? I have suggested that sex determination may be such a function². The observed dosage compensation of *G6PD*, *HPRT*, *PGK* and such other X-linked housekeeping genes would then be, in the evolutionary sense used by Maynard Smith³, an effect of X inactivation, not its function. This model requires that a distinction be made between dosage compensation at the level of the phenotype and that which might render the effective copy number of particular X-linked genes equal between XX and XY genotypes. Details are given in ref.2.

In *Drosophila melanogaster*, the Bridges ratio, X/A, is central to both sex determination and X-chromosome dosage compensation. Sex-specific lethal mutations such as *mle* and *Sx^m* affect both sex determination and dosage compensation, demonstrating that some genetic elements are common to the two processes^{4,5}. Thus it appears that sex determination and dosage compensation may be two facets of the same regulatory process. A plausible genetic regulatory model has been proposed for intracellular measurement of the X/A ratio and the remarkable phenotypic effects of certain of these mutations⁶. An

attempt has been made to interpret mammalian X inactivation in terms of this model, on the assumption that X inactivation also is based on some type of ratio measurement or intracellular counting of chromosomes⁷.

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The Grand Menhir re-examined

SIR—Commenting on P.G. Bahn's alerting us¹ to C. T. LeRoux's discovery that the capstones of three tombs in Brittany once formed a single decorated 14m long menhir, one of the decorations being a carving of a *hache-charrue* (axe-plough), I suggested in *Scientific Correspondence*² that Le Roux's discovery requires a re-examination of the date and function of the over 20m long now fallen and broken menhir called the Grand Menhir Brisé. My suggestion assumed the validity of the assertion first made by R.S. Minot³ and then by E. Twohig⁴ that there is also a *hache-charrue* carved on the latter menhir.

I recently examined the Grand Menhir Brisé and could not discern a *hache-charrue* carved on it even after I checked my observations against the sketch by Minot in his 1965 article and against the small scale drawing furnished to me by Twohig. There are unevennesses on the surface of the granite of the second fragment of the Grand Menhir Brisé (counting the largest fragment as the first fragment) which may be interpreted as possibly depicting an axe head, but there is no indication that these are man-made rather than natural, and the presence of the rest of the alleged *hache-charrue* seems to depend upon the imagination of the beholder⁵ (See *Hamlet*, Act 3, Scene 2, "Shape of cloud").

Twohig, in the course of her careful and thorough research, did not always find carvings reported by Minot to be genuine, (personal communication) and Minot's sketch of the alleged *hache-charrue* differs from Twohig's drawing in substantial ways, including not showing a loop on the haft. Because of its size and weathered nature, Twohig did not make a rubbing, photograph or full-scale tracing of the alleged carving, and the small scale drawing on which she bases the drawing in her book reflects what she saw but does not

necessarily record what others will see.

One further observation should be made. LeRoux interprets the three granite fragment now serving as capstones of La Table des Marchands, the tomb of Gavrinis, and the tumulus of "er-Vinglé", as having once formed a single carved menhir. R.J.C. Atkinson suggests that the three granite fragments determined by LeRoux to once have been a single standing stone was a very long capstone which was later broken, its fragments then used as capstones in three separate tombs (personal communication). A very long capstone would be consistent with the shape of the stone when unbroken, and we know that dolmens were constructed which could utilize very long capstones, such as Le Grand Dolmen at Bagneux (Maine-et-Loire).

Atkinson points out that menhirs, unlike upright stones and capstones used in tomb construction, do not have carvings on them, except, in rare instances, for some markings at the base, and certainly not halfway up the menhir. In such event the date of construction of the Gavrinis tomb of about 5200 to 5000 BP would be irrelevant to the determination of the date of erection of the Grand Menhir, and a possible date of about 3600 BP initially proposed by Alexander Thom⁶ and A.S. Thom in connection with their lunar observatory hypothesis would not be affected by LeRoux's discovery.

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Nature's Guide to How Britain Runs its Science

THE latest edition of this popular wallchart is now available. The guide provides a comprehensive survey of the administration of British science, showing at a glance the functions, key personnel and finance of government departments, public and independent bodies, and the research councils. It has been thoroughly updated to take account of the many changes in structure and personnel that have occurred since the previous edition was published in 1983.

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Intellect in evolution

Douglas J. Futuyma

Evolution: Essays in Honour of John Maynard Smith. Edited by P.J. Greenwood, P.H. Harvey and M. Slatkin.

Cambridge University Press: 1985. Pp.328. £30, \$49.50.

By the fertility and originality of his thought, the variety of his interests and contributions, and the influence of his effervescent personality on his colleagues and students, John Maynard Smith has taken his place as one of this era's leading evolutionary biologists. His empirical and theoretical contributions have included functional morphology, *Drosophila* genetics and cytology, the genetics of developmental patterns, the evolution of senescence, the theory of polymorphism, speciation, evolutionary rates, and kin and group selection, the evolution of sex, and the evolution of behaviour. This *Festschrift* in honour of Maynard Smith on his imminent retirement from Sussex University therefore marks the end of an era.

Maynard Smith, perhaps more than any other single individual, has embraced both major strategies of explanation of evolutionary change: the formal theory of population genetics, including natural selection; and the phenotypic theory of adaptations. This second theory of adaptive strategies is largely devoid of genetics, but attempts to predict, for real phenotypic characters, the ordering of fitness values that appear as abstract constructs in population genetics. Both of these approaches, between which there is considerable tension, are represented in this collection.

The power of population genetics is revealed in Slatkin's theoretical treatment of the role somatic mutation might play in defending plants against attack by creating genetic mosaics (he concludes it will usually have little effect), and by Bengtsson's review of the number of underdominant loci required to establish a sterility barrier to gene flow between incipient species (generally many are required). These essays exemplify Lewontin's point that the role of population genetic theory is not to predict evolution, but to delineate the prohibited and the possible. Lewontin is less encouraging on the ability of theory to explain observations (for example to specify causes for gene frequency distributions), but is hopeful that variation at the nucleotide level may "give a finer structure of genetic detail corresponding to the detail that is built into the theory". Yet the theory includes elements such as population size, gene flow and linkage disequilibrium that surely will never be known in such detail, so the scope for such optimism may be rather narrow.

Representing the population genetic approach to adaptations, Felsenstein provides an intriguing distillation of theories of recombination, with some surprising conclusions (for example, the sib-competition model of Williams is a special case of Fisher and Muller's theory). Michod and Sanderson develop a theory of behavioural structure that obviates the



Maynard Smith: a view of both major strategies of explanation of evolutionary change.

need for kin selection as a necessary explanation of cooperation. Rose reviews his work on the genetics of senescence.

Strategic analysis has become one of the chief tools in the study of adaptations, and Maynard Smith's introduction of the evolutionary stable strategy (ESS) concept is surely one of his most important contributions. The pitfalls lie not so much in the theory, which like population genetics theory varies in the realism of its assumptions, but in the testing, especially by the comparative method. Charnov shows the power of ESS theory to predict sex ratio and related phenomena, and uses the comparative method to good advantage. Packer and Pusey make a fairly convincing case for the ability of ESS theory to predict the outcome of asymmetrical contests in social mammals.

But observations do not always conform to the theory, as both Clutton-Brock and Parker and Hammerstein note, or they may conform for the wrong reasons. For example, Silvertown cites the composite *Hypochoeris glabra* in support of the hypothesis that the proportion of far-dispersing fruits should increase with fruit number because of density-dependent competition. But because near-dispersed

fruits develop only on the circumference of the capitulum, the conformity to expectations is a necessary allometric consequence of the ratio of circumference to the area.

In other instances, the sensitivity of the test is inadequate. For example, D. Charlesworth attempts an admirably careful test of the hypothesis that dioecy has evolved in angiosperms as an outcrossing mechanism, by seeing if it is negatively correlated with self-incompatibility across families and genera. The data are inadequate for the test, but even if they were adequate, a census of this kind is not sufficient, because a phylogenetic analysis is required in each case to infer whether dioecy arose in a self-compatible or self-incompatible ancestor.

We are now all aware of the excess of overenthusiastic adaptationism, and of the difficulties of testing strategic theories of adaptation. But the theory itself may prove to have important limitations, as Parker and Hammerstein point out in an excellent review of evolutionary game theory. For example, realistic extensive games in which the current decision depends on previous events typically have multiple stable solutions. Thus the historical contingency of evolution is inescapable, even in optimality models. Parker and Hammerstein also note that the necessary synthesis of strategic theory with population genetics has not yet occurred and may prove difficult – as the schism within this book bears witness.

Among the remaining essays, some fall within these two approaches: Seger presents a very restricted genetic model of sympatric speciation, León theorizes on seed germination, Gittleman reviews possible advantages of aggregated placing of young by mammals, and Greenwood and Wheeler offer a highly speculative physiological explanation of sexual size dimorphism in homeotherms.

A potpourri of other contributions includes an exemplary exercise in adaptive hypothesis testing by Harvey and Ralls concerning body size in weasels, an intriguing failure by Bradbury, Vehrencamp and Gibson to find a mechanistic basis for the variance in male reproductive success in lek-forming species, an elaboration by Stenseth of Maynard Smith and Stenseth's theory of lag-load and evolutionary rates, and a benediction by May.

This well-edited volume offers a nicely balanced overview of some of the most active areas of investigation of adaptation. Like most honorary volumes, it is neither deep nor comprehensive, but several of the essays, as I have described, are important and thoughtful, and will surely be cited widely. □

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Action at a distance in public affairs

Joseph Haberer

Einstein in America. By Jamie Sayen. Crown: 1985. Pp.340. \$17.95.

OF Einstein it may be said that "fame was the spur" for his life-long involvement in matters relating to political and social issues. In 1920, with his sudden and unanticipated emergence as a world figure, he recognized that his name and celebrity status could be used as a means to shape public opinion and influence those who made decisions on issues with which he was concerned: notably, war and peace; Zionism and the Jewish people; social and economic justice; the threat of fascism and Hitler; the responsible and humane use of scientific knowledge.

Einstein's fame took hold first and especially in America. It is his relationship to the country of his eventual adoption that is at the centre of this biography. In 20 chapters Sayen explores thoroughly and with great acumen the many aspects of Einstein's experience with and in America: from the first journey in 1921 (with Chaim Weizmann, to raise money for the Hebrew University and publicize the Zionist cause) to his last effort in 1955 with Bertrand Russell through a Manifesto to warn against the dangers of the arms race. Most of the book deals with the years after Einstein settled in America in 1933, but three chapters cover the years prior to his arrival and provide an excellent background to the American phase.

Sayen's book is the first to deal primarily with Einstein's various political activities during his mature years, and since there was no intention of writing a complete biography little attention is given to Einstein's scientific work (Abraham Pais's *Subtle is the Lord*, published in 1982 by Oxford University Press, has done this superbly). Sayen demonstrates a thorough grasp of the Einstein materials and literature; evident throughout is his careful and impressive use of the large correspondence from the Einstein Archives. Earlier biographies, most notably Ronald Clark's *Einstein: The Life and Times* (World Publishing, 1971) were clearly deficient in this regard, and are quite misleading on several important aspects of Einstein's political life.

The Einstein that emerges in these pages is a brilliant scientist, as one would expect; a man with a finely honed sense of social responsibility; a person of great warmth, kindness and modesty. We know however, remarkably little about his deeper self and he seems to have gone to considerable lengths to keep it that way. But we know of some problems, most not-

ably his life-long failure to establish intimate personal relationships. Sayen shows Einstein with some of his flaws, and thus avoids the all-too-frequent hagiographic accounts that have come our way.

There is a widely held view that Einstein's political perceptions and activities were those of a man filled with good intentions but characterized by a rather naive grasp of politics and social issues. Sayen lays this myth to rest. Einstein clearly made it his business to be well-informed on those matters on which he took a public stand or in which he became otherwise engaged; he would not, for example, sign petitions, write references or support causes unless he had satisfied himself thoroughly that the facts were such that he could in good conscience do so. He was fully aware that his name was his credit, and he made every effort not to squander it. To be sure he made some mistakes, but he learned quickly to be more careful, and it is remarkable how seldom he erred in his assessments of people and events.

Einstein was the progenitor of the scientist as a publicist for the causes with which he identifies. He wrote (for a scientist of his day) extensively in the press on social and political subjects, and kept up a flow of correspondence in which he addressed important questions of the time. Sayen's book demonstrates that Einstein's understanding of the political and social realities of the twentieth century, as well as his underlying philosophical positions, were much keener and showed a better grasp of the emerging forces than those of most of his critics. For example, his attitude towards the Soviet Union was sympathetic towards its socialist objectives, but from the early 1920s — long before the Stalinist excesses — he was quite aware of the human rights abuses. He refused in the 1920s to sign petitions attacking right-wing excesses unless they included criticism of those of the left also. But he argued in the post-1945 period that an accommodation with the Soviet Union was imperative for a lasting peace, though he recognized the obstacles on both sides that stood in the way. His reluctance to criticize the Soviet Union publicly during the Cold War period was based on complicated reasoning, but came down essentially to an unwillingness to add fuel to the anti-communist hysteria in America.

Although Einstein understood politics, he appears to have thoroughly disliked the activity and the process. He took positions, but avoided choosing parties, or becoming a partisan. Perhaps he resembled somewhat the platonic philosopher-king, who reluctantly, but out of perceived necessity, enters the tainted and turmoil ridden world of the political. He sought to influence while keeping his distance. □

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Close considerations

Jack Cohen

Biology of Fertilization. Edited by Charles B. Metz and Alberto Monroy. *Academic*: 1985. Three volumes, pp.391, 475 and 469. \$75, £75 each volume.

IN THE late 1960s, a number of people, myself among them, moved from other fields of developmental biology into research on fertilization, partly because electron microscopical techniques were dramatically illuminating the magic moments of membrane fusion but also because mammalian gametes were just becoming accessible to experiment. *Concepts of Fertilization*, edited by Monroy and Metz and published in 1967, was a guide book to the territory for us. That collection of authoritative, and revelatory, review articles by all the "big names" put the subject in context and provided both opinion and forecasts; it did not have the now-fashionable tone that "for each expert there is an equal-and-opposite expert", with each disagreement referenced and written in the dense prose which now seems mandatory for the professional review. The editors were daring, for that time, in assembling biochemists, botanists and electron-microscopists, as well as fertilization specialists, and in suggesting that work upon bacteria, algae and *Paramecium* could illuminate sea urchin and mammal fertilizations.

This new collection, which comes in three volumes entitled *Model Systems and Oogenesis*, *Biology of the Sperm* and *The Fertilization Response of the Egg*, looks very different. We have all got older, and we now know much more. But beyond that I believe there is a much more basic and serious difference. As an area of science develops, knowledge should not simply accumulate; generally accepted theories should encompass large areas of previously disparate observations and bring them into a coherent framework. New textbooks then reflect this coherence in their examples for students. There have been several attempts in the area addressed by this collection, of which Austin and Short's series *Reproduction in Mammals*, now going through a second edition, has probably been the most successful.

For me, this new collection of reviews fails because it gives only sporadic indications of the larger view. The density of information, argument and referencing of most of the articles is appropriate to the subjects they consider, and these articles could well have been found in the *Advances in . . .* or *Current Topics in . . .* sort of book. What is missing is the ultimate rather than the proximate consideration. All the authors, even those considering polyspermy and its prevention, write of

the sperm and the egg, and the failure of the vast majority of gametes to fertilize is never invoked in arguments about the few which do and the mechanisms they seem to use. Similarly, the enormous variety of mRNA elaborated by the oocyte's diploid (maternal) genome and stored in mysterious ways for use by the cleaving embryo is explained as the proximate mechanism to supplement the mRNA-synthetic abilities of the single zygote nucleus, in the immense egg cytoplasm, until such time as the nuclei have multiplied. Surely the theoretical importance of early development being controlled by maternal genome, only mentioned by Monroy, could have been expanded somewhere? The titles of the individual reviews often promise such a general approach — "Nucleo-cytoplasmic interactions . . .", "Activation of DNA synthesis . . .", "Regulation of mammalian spermatogenesis", "Vertebrate sex determination" — but each focuses down on one problem and doesn't usually generalize in the Discussion section either; there are too many "More work is needed before meaningful generalizations . . ." conclusions. If this is so, then this is not the time to review that subject.

The reviews seem mostly to have been written in about 1981, and to have been updated in 1983–1984; later references are nearly always to the author's own work. This is inevitable in such a grand compilation, but it makes the previous criticisms much more serious. Generalities, indeed prejudices, of workers of this eminence will usually keep their interest for

some years, but these minor issues lose immediacy much sooner. Particularly unfortunate in this context is the absence of the inositol triphosphate (IP3) story of 1984, which provided a new second messenger with close affiliations to the cell membrane; it rendered incomplete nearly all of the controversy about Ca^{++} , much of that about membrane receptors of egg or sperm, and all of that about phospholipid involvement in sperm maturation. An attempt by Whitaker and Steinhardt to make good this particular deficiency by way of an addendum to their Chapter 5, printed at the end of Vol. 3, only succeeds in emptying this can of worms among all the other contributors: tangled discussions of cytoplasmic pH, Na^+/H^+ exchange and egg activation/cortical reactions, and even the bindin story, are shown to have been incomplete, needing identification of this crucial thread. If theories had been proposed, IP3 would have added mechanism; as it is, for most of the articles subsequent discovery invalidates much of their content.

There is certainly a place for this collection in university libraries, and even in the more specialized collections of directors of (rich) *in vitro* fertilization units, while individual articles will be very valuable, especially for research students. But what a pity it is that they will miss the real expansion of mental horizons which we gained from the first edition. □

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Products of history

Janet Browne

Seeds of Change: Five Plants that Transformed Mankind. By Henry Hobhouse. Sidgwick & Jackson: 1985. Pp.245. £15. To be published in the United States later this year by Harper & Row.

POTATOES are not usually given much thought beyond the kitchen table, yet in Henry Hobhouse's *Seeds of Change* they take on a starring role. Rightly so, for these humble tubers have had a dramatic part in world events. The blight of 1845 meant nothing less than starvation for the Irish nation; economic crisis erupted in Britain, forcing rapid tax reform, repeal of the Corn Laws and a temporary halt to restrictive trading practices; and on a larger scale, one more wave of hopeful settlers emigrated to the United States where they then went on to become a recognizable force in the shaping of modern America.

There are other plants, other commodities that have changed the course of his-

tory and, of these, Hobhouse selects a further four to illustrate his general theme that plants matter more than one might think. Sugar cane in the West Indies, a crop supported by an economic and social superstructure of plantations and slavery, was, by his account, responsible for the infamies of the slave trade and for nearly half of all the seagoing effort, naval and civil, of western European countries before 1830. It gave Britain commitments in the Caribbean that Hobhouse feels she could well have done without (and an irredeemably sweet tooth). Cotton, on the other hand, made the American south rich and confident. This too was a cash crop based on slavery, contributing to a lifestyle that was ripped apart by the Civil War and yet, ironically, feeding the first great industrial revolution with raw material for newly mechanized processes of textile production.

Then there is tea. The British tea trade with China during the eighteenth century heralded not only imports of porcelain ("china"), opium and redesigned "clipper" ships, but also a fight for economic supremacy with the Dutch East India Company, encouraging Britain to concen-

trate primarily on India, and the opium wars of 1840–1842 that finally opened up two British footholds in China — Shanghai and Hong Kong. Last of all, although first in his book and undoubtedly Hobhouse's most interesting chapter, there was the hunt for quinine, the bark of a South American tree which prevents and cures malaria, thereby allowing the opening up of great tracts of central Africa and India to European colonial powers.

There is much here that will be fresh, surprising and intriguing to most readers whatever their background. The stories of these staple products are extraordinarily rich, containing within them all kinds of repercussions that echo through to the

IMAGE
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REASONS

Wartime exhortation in the Daily Express of 18 January 1943. The cartoon is reproduced in the revised impression of The History and Social Influence of the Potato by Redcliffe Salaman, which has been edited and is introduced by J.G. Hawkes. Publisher is Cambridge University Press, price is hbk £35, \$49.50; pbk £12.50, \$16.95.

present day. But for professional historians, perhaps for scientists too, the book is hampered by Hobhouse's all-pervading sense of moral outrage at the activities of our ancestors, and his implicit assumption that things are better now. Perhaps his forthcoming sequel (on corn, oil and wine) will be less Anglocentric and less judgemental. Many readers will surely wish for something more of an analysis of the actual causes involved in, for example, the colonization of India; the ability to control malaria through quinine, critical as it may have been, is hardly a reason for India's emergence as the jewel in the British Crown, any more than it is an explanation for the scramble for Africa. Hobhouse's breezy good sense is no match for the deeper causes lurking here. □

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Connecting with the invertebrates

Adrian Horridge

Model Neural Networks and Behavior. Edited by Allen I. Selverston. Plenum:1985. Pp.548. \$69.50, £66.03.

THE scope of this book is less than the title suggests and limited largely to central nervous mechanisms in invertebrates. More than 20 of the contributors are from the US west coast and the same number work during summers at Wood's Hole. The dedication to Graham Hoyle is apt. In some sense it represents a panegyric of collective achievement as the topic has developed several important cutting edges, because, in Selverston's words, these invertebrate systems have "characteristics especially suitable for answering specific questions".

The qualitative analysis of real connections between actual neurones in favoured invertebrate preparations, and the origin of these patterns in development and inheritance dominate the scene. Fundamentally the book is an expression of the reductionist approach, in which analysis goes from detail to detail, down to actual mechanical causes by every available technique. The chase leads into topics such as the "activity-dependent enhancement of synaptic facilitation" as the mechanism at the point where learning actually occurs, to long-lasting effects of synaptic modulators, to cyclic AMP and calcium ions. In the growth studies, the editor has selected accounts of marking membranes with antibodies which reveal that neurone surfaces differ chemically, but the reductionism is non-quantitative, an interesting paradox.

With this type of analysis, an important feature is the commonality of the results. Almost every one of the 27 papers concentrates on a different preparation but the results seem to be comparable. We begin to understand that there are only a limited number of mechanisms, presumably based on a limited number of conserved DNA sequences, which generate channels, transmitters, receptors on membranes, hormones, and units in a construction set. After a century of effort, we understand many of these components; by contrast, we are just at the threshold of the chemical mechanisms that bring and hold the pattern of connections together. The final chapters on neurogenetics are excellent snippets of a fast-moving and fascinating topic.

As an exercise to demonstrate the importance of invertebrate preparations to our understanding of nervous systems, the volume is a great success. Identifiable neurones, large enough to be dissected

out singly and analysed for chemical content, have pushed our understanding much further than is possible with vertebrates.

That statement applies to much besides the contents of this book, notably to sense organs and sensory processing. For this volume has nothing on information flow, signal-to-noise ratios, or on the optimization of the neural networks. The software is all molluscan: the hardware chitinous. Those recording from anonymous mammalian neurones of medical interest get on-line computers whereas those slogging to elucidate the exactly identified invertebrate neural circuits pursue a task which one of them rightly recognizes as "a long and irksome process". But neural networks, surface-specific molecular interactions in growth, and polypeptide sequences are also topics where an enormous amount of tedious fact-finding must be done before elegant generalizations can be reached, simply because the human understanding has no ready-made models for ordering these new bodies of data.

There are seven chapters dealing with neural circuitry, nicely spread between leech, molluscs, locust flight motor and the lobster pyloric ganglion. Again this is essential foundation work, defining the components, that opens up studies of growth, learning, plasticity, transmitters and membrane biophysics. Later sections in the book deal with all these topics. Development studies, cellular chemistry and

membrane biophysics are again spread between insects, a mollusc and leech. Analysis of learning in circuits of identified neurones is restricted to molluscs, where work has consolidated over the past decade. Even neurogenetics now spans three phyla.

Unfortunately the presentation is often lack-lustre; lacking any introductory concessions, lacking spice, lacking a real philosophical backing, and certainly lacking a historical perspective or even a decent index. Essentially a result of lack of thought, it is today characteristic of much scientific writing done as an account of ongoing activity, out of date in five years. I suspect many of the circuits described are oversimplifications. Nowhere in the book is there a consideration of how to analyse neurone function. I am frustrated however that my own student, whom I have tried to interest in this problem, states that "this is the wrong type of question".

There are 60 contributors, among them most of the best specialists in the field, and there are many excellent contributions. The whole book is a splendid summary of ongoing activity at an expert level contributing to the understanding of many aspects of all nervous systems. The book should be read by all who try to make sense of how central nervous systems are really put together in the non-mathematical non-engineering sense. □

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Delayed responses

P.F. Baker

Calcium in Biological Systems. Edited by R.P. Rubin, G.B. Weiss and J.W. Putney. Plenum:1985. Pp.737. \$89.50, £85.03.

COMING to grips with the many roles of calcium is just another task the modern biologist must master. This volume is based on the proceedings of a "calcium theme" included in the 67th Annual FASEB meeting held in Chicago in April, 1983. It includes 80 contributions divided into three sections: metabolic and functional aspects, regulation of muscle contractility, and nutritional and pathophysiological aspects of calcium action.

In general, the choice of contributors and contributions — which range from calcium chemistry and biochemistry to clinical medicine — are excellent, although readers would have benefited from a few more overviews setting specific topics in perspective. A far more important criticism, however, is that the meeting was held in 1983 and the book only appeared in 1985, two whole years later.

For a field that is changing as fast as calcium such a delay is unacceptably long and, unfortunately, robs the book of much of its value. This is very sad as the book contains a great deal that would have been highly topical in 1983 or even 1984, but by now has largely reached the scientific community by other means. Nevertheless, it says much for the editors' choice that there is still material of considerable interest and the volume clearly delineates the ubiquitous and pivotal role of calcium in diverse physiological and pathological states. It is only a pity they did not convince their publishers that for those intending to purchase a book of this type, its value is inversely related to the delay between the meeting and its publication.

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• A recent volume in Humana's *Contemporary Biomedicine* series is *Calcium and Contractility: Smooth Muscle*, edited by A.K. Grover and E.E. Daniel. The book is a collection of reviews by well-known figures in the field and contains, says the publisher, "Incisive synthesizing summaries of the powerful outpouring of research on cell contractility today". Price is \$64.50 in the United States, \$74.50 elsewhere.

Rim syncline volume estimation and salt diapirism

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Quantitative studies of salt structures are based on the assumption of a simple relationship between excess sediment volumes and the volumetric growth of salt structures. This assumption is inadequate and, instead, this article shows how the excess volume relates to the volume swept out by the deforming sediment surface and the moving salt/sediment interface. The use of this new basis for volume reckoning is demonstrated, and its implications for waste disposal, hydrocarbon exploration and tectonic subsidence studies are emphasized.

SALT pillows and salt diapirs occur in great numbers in several sedimentary basins. The movement of salt dramatically changes the depositional geometry of its enclosing basin, creating, for example, structural traps for hydrocarbons. Salt structures are also considered suitable sites for the disposal of high-level nuclear waste. Thus, there are compelling reasons for studying such structures.

Studies of natural salt diapirism are built on the classic work of Trusheim^{1,2} who demonstrated how the history of a diapir can be unravelled by observing the location and timing of excessive sediment thicknesses, assuming these to be caused by subsidence over a depleting source layer. The main features of Trusheim's model are illustrated in Fig. 1 (b, c). In the pillow phase¹, excess sediment volumes are deposited in the primary rim syncline (V_{rs}^1), far from the centre of the structure (Fig. 1b). The excess volume (V_{ex}) is defined as the rim syncline volume (V_{rs}^1) minus the 'regional normal' (V_{rn}). 'As measured' sediment volumes must be corrected for compactional effects. The amount of finitely displaced salt (V_{st}) is the volume of salt above the original source layer (of thickness s_0). The diapiric phase rim syncline (V_{rs}^2), the secondary rim syncline¹, is characterized by excess volumes adjacent to the diapir (Fig. 1c). It is overlain by an only slightly structured rim syncline (V_{rs}^3) of the post-diapiric phase, the tertiary rim syncline. Trusheim's model is qualitative, but its quantitative potential was realized "Das Volumen des akkumulierten bzw. extrudierten Salzes entspricht in jeder Phase dem der Sedimente, die diese Bewegungen kompensieren, sofern man die epirogene Absenkung subtrahiert" (ref. 1, p. 145). Although Trusheim's 'volume rule' has been used by several authors³⁻⁷, there has been no systematic discussion of volume estimation. This article aims to start such a discussion and, using an example, to demonstrate its relevance. The volume discussion is generally valid, but the diagrams illustrating it (Figs 2, 3) are two-dimensional, that is, areas sum in the figures as volumes in the general discussion.

Pillow phase

Analogous with experimental diapirism⁸, it is hypothesized that a salt pillow outlines the growth stages (Fig. 2a) whose surface expression is a laterally extending sink and narrowing source. The salt and sediment displacement volumes are laterally extended and do not overlap vertically, so that the following equation can be considered valid for volumes summed over the entire salt intake area (Σ_a) and over time (Σ_t)

$$\Sigma_a \Sigma_t V_{sk}^1 (= \Sigma_a \Sigma_t V_{sc}^1) = \Sigma_a \Sigma_t V_{std}^1 (= \Sigma_a \Sigma_t V_{std}^1) \quad (1)$$

where the superscripts designate the pillow phase. Complexities in volume estimation arise because such physically real volumes as, for example, the sink volume, have to be related to the measurable excess volume, which is an operationally defined construction.

The growth stages envelop an 'overlap volume', acting first as a sediment source and then as a sediment sink (surface overlap

volume, V_{so}). A corresponding overlap volume (displacement overlap volume, V_{do}) is enveloped by the subsurface salt-sediment interface. Within the displacement overlap volume, sediments are first displaced by salt, then salt by sediments.

The overlap volumes 'measure' the path of the structure (Fig. 2a). Whether the surface overlap volume is reflected in the excess rim syncline volume depends on the regional rate of sedimentation. Two extremes can be envisaged.

First, when the regional rate of sedimentation (v_{sd}) is zero (Fig. 2b), uplift within the surface overlap volume leads to erosion of the pre-pillow series ('pre' of Fig. 2a), so that the cumulative excess sediment volume is equal to the cumulative sink volume (Fig. 2a, sum of arrowed prisms). This, in turn, is larger than the volume of finitely displaced salt (V_{st}) by an amount equal to the surface overlap volume (Fig. 2a), so that

$$\Sigma_a \Sigma_t V_{ex}^1 = \Sigma_a \Sigma_t V_{sk}^1 = V_{st}^1 + \Sigma_a \Sigma_t V_{so}^1 \quad (v_{sd} = 0) \quad (2)$$

Second, when the regional rate of sedimentation exceeds the rise rate of the structure (v_{up}), the uplift within the surface

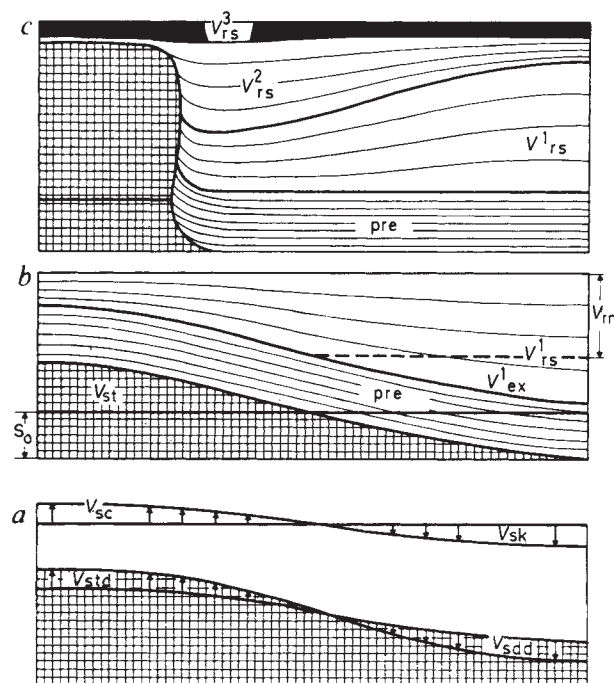


Fig. 1 Volume terms and Trusheim's model¹. a, Salt displacement volume (V_{std}) and sediment displacement volume (V_{std}) overlain by sediment source (V_{sc}) and sediment sink (V_{sk}), respectively. b, Trusheim's pillow phase with the primary rim syncline (V_{rs}^1) overlying the pre-pillow series (pre). V_{ex} , Excess rim syncline volume; V_{rn} , regional normal volume; V_{st} , volume of finitely displaced salt. c, Trusheim's diapiric and post-diapiric phases. V_{rs}^2 , Secondary rim syncline; V_{rs}^3 , tertiary rim syncline; source layer thickness, s_0 .

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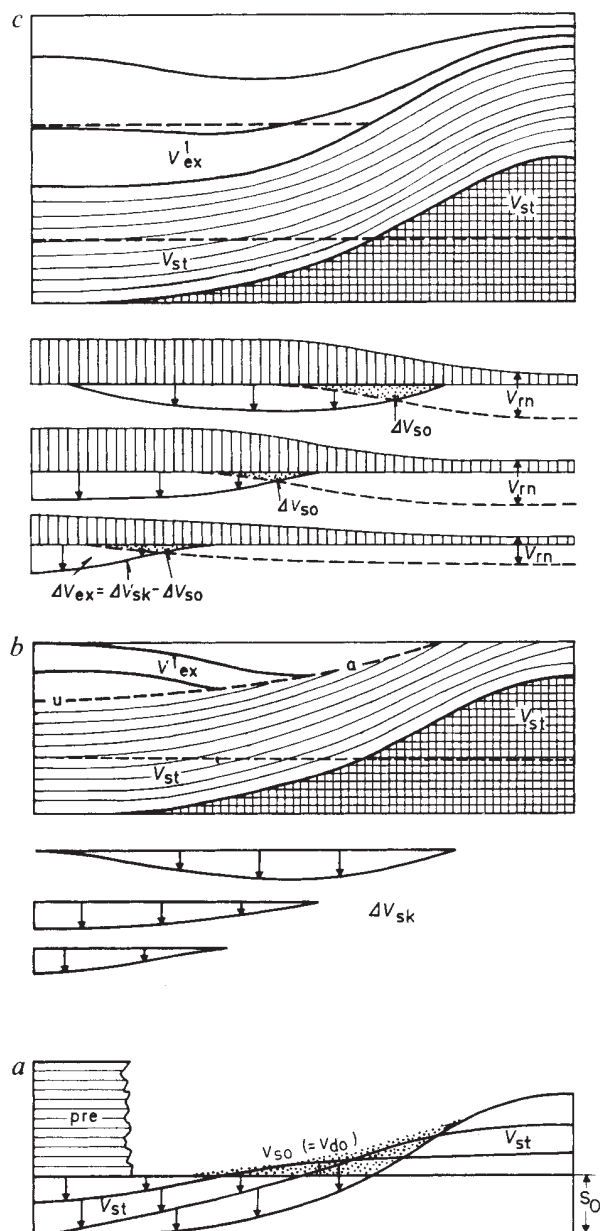


Fig. 2 Pillow phase volume estimation. *a*, V_{so} , surface overlap volume; V_{doz} , subsurface displacement overlap volume. Pre-pillow series (pre) and source layer thickness (s_0). *b*, Pillow formation with no regional sedimentation. The excess volume (V_{ex}^1) is equal to the sink volume ($\Sigma \Delta V_{sk}$) but larger than the displaced salt volume (V_{st}). Note the angular unconformity (au) between rim syncline layering and the pre-pillow series. *c*, Pillow formation with the regional rate of sedimentation larger than the salt uplift rate. The excess volume (V_{ex}^1) is smaller than the sink volume ($\Sigma \Delta V_{sk}$) by an amount equal to the overlap volume ($\Sigma \Delta V_{so}$) but equal to the displaced salt volume (V_{st}). This limiting case is similar to the pillow phase of Trusheim's model (see Fig. 1b). Note the absence of angular unconformities. Arrows define cut-out volumes.

overlap volume causes a regional sedimentation (Fig. 2c, vertical ruling) less than normal (V_{rn} , Fig. 2c). When deriving the excess volume by subtracting a constant regional normal (Fig. 2c, vertical ruling plus stippled) from the rim syncline volume (the "epirogene Absenkung" of Trusheim¹), the excess volume becomes (see Fig. 2c)

$$\Sigma_a \Sigma_t V_{ex}^1 = \Sigma_a \Sigma_t V_{sk}^1 - \Sigma_a \Sigma_t V_{so}^1 = V_{st}^1 \quad (v_{sd} > v_{up}) \quad (3)$$

Only in this case will the compaction-corrected excess volume be equal to the finite amount of displaced salt. For the pillow

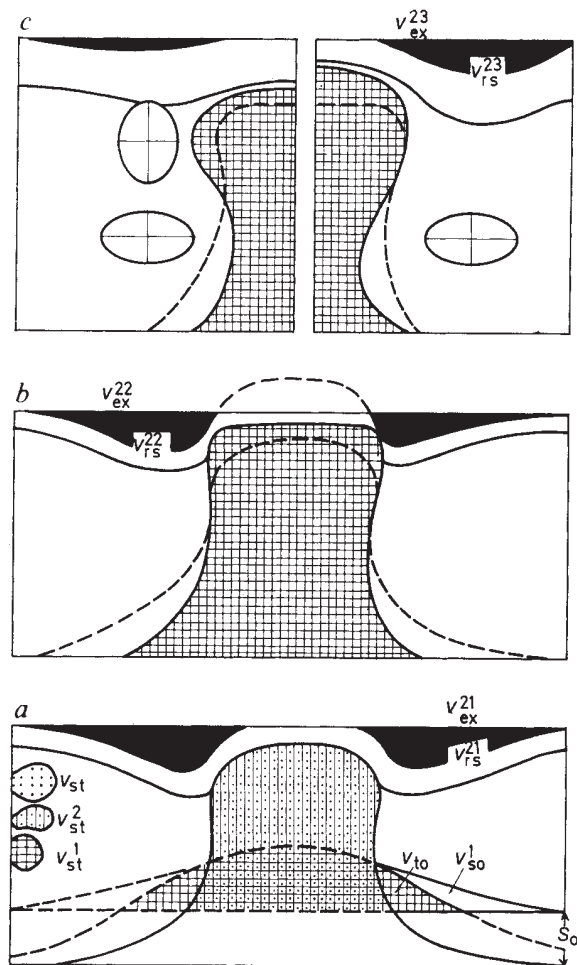


Fig. 3 Three modes of the diapiric phase. *a*, Intrusion mode; its rim syncline (V_{rs}^{21}) and its excess volume (V_{ex}^{21}). V_{so} , Surface overlap volume; V_{to} , transition overlap volume; V_{st} , salt volumes. *b*, Dissolution mode; its rim syncline (V_{rs}^{22}) and its excess volume (V_{ex}^{22}). *c*, Overhang mode; its rim syncline (V_{rs}^{23}) and its excess volume (V_{ex}^{23}). Right, overhang formation by necking. Left, overhang formation by combined necking and top spreading.

phase, in general, equations (2) and (3) combine to give

$$V_{st}^1 + \Sigma_a \Sigma_t V_{so}^1 \geq \Sigma_a \Sigma_t V_{ex}^1 \geq V_{st}^1 \quad (4)$$

As illustrated in Fig. 2, the internal structures of the primary rim syncline can be expected to differ significantly in the two cases, although the sink volumes of both cases, Fig. 2b, c, (triangular prisms with arrows) are equal. In the first case, angular unconformities between the layering of the primary rim syncline and the layering of the pre-pillow series develop. In the second case, the primary rim syncline will be characterized by converging and diverging layering. It should therefore be possible to decide which side of equation (4) the excess sediment volume of a specific structure approaches.

Diapiric phase

Trusheim suggested that the transition from the pillow to the diapiric phase was discontinuous, the pillow formation leading eventually to a fault-controlled breakthrough. If the diapir develops without significant lateral movements, overlap volumes are not formed. In this phase, the excess volume therefore corresponds to the sink volume. But other types of volumetric discordances may develop. They can be described with reference to three 'modes' of diapirism (Fig. 3). Real diapirs are likely to alternate between the intrusion and dissolution modes after

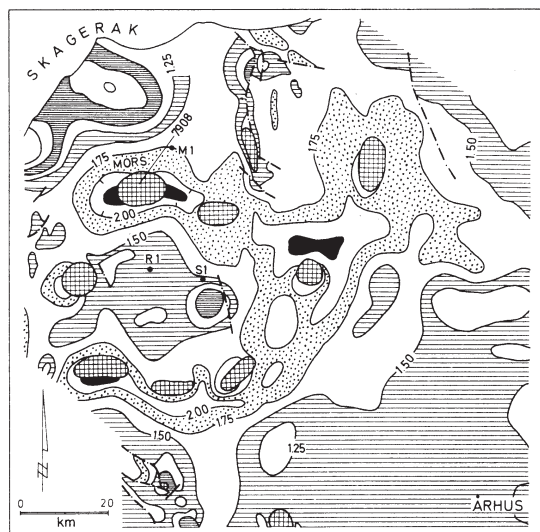


Fig. 4 The Danish onshore salt structure province. Cross-hatched, salt diapirs. Contours, two-way-travel time in seconds to the Gassum Formation reflector. The Gassum reflector is undisturbed under the Århus-Silkeborg Flat (west of Århus)¹⁹ and under the Rødding Flat (south of the well R1)¹⁴. Deep wells mentioned in the text: R1 (Rødding 1), M1 (Mors 1) and S1 (Skive 1). 7908, seismic profile discussed in the text. Simplified after a map by Baartman, Olsen and Woodward (in ref. 20).

initial intrusion, and overhang formation may occur in combination with both intrusion and dissolution.

In the 'intrusion mode' (Fig. 3a) the following equalities hold

$$\begin{aligned} \Sigma_a \Sigma_t V_{ex}^{21} &= \Sigma_a \Sigma_t V_{sk}^{21} (= \Sigma_a \Sigma_t V_{sc}^{21}) \\ &= \Sigma_a \Sigma_t V_{sdd}^{21} (= \Sigma_a \Sigma_t V_{sd}^{21}) \end{aligned} \quad (\text{intrusion mode}) \quad (5)$$

Because, by definition, no material transfer occurs across the fault plane, the sink volume must also be equal to the displacement volume (provided intrusion is fault-controlled). The first superscript designates the diapiric phase, the second, the mode.

For some combinations of overburden thickness, regional sedimentation rate and diapiric rise rate, diapirism leads to breakthrough (Fig. 3b) to the surface (extrusion) or to near-

surface dissolution. In both cases, salt is dissolved, so that the surface source and sink volumes are no longer equal. The volume estimation formula for the 'dissolution mode' of diapirism must include this inequality which can become significant in two respects. If distant sources cannot supply material, lack of a nearby sediment source will lead to a deepening sink. In this stage, depositional patterns, which are entirely different from the regional pattern, may develop. Under some circumstances, petroleum source rocks can be anticipated in the rim syncline, in other conditions evaporite (re)deposition⁹. In this mode, salt diapirism may have a generic influence on sedimentation, and not the merely moderating influence suggested recently⁶. The discordance between source and sink volumes may also lead to delayed filling of the sink, in which case the possibility of excess sediments post-dating the age of the sink arises. The complete volume formula is therefore

$$\begin{aligned} \Sigma_a \Sigma_t V_{sdd}^{22} &= \Sigma_a \Sigma_t V_{sk}^{22} > \Sigma_a \Sigma_t V_{sc}^{22} \geq 0 \\ \Sigma_a \Sigma_t V_{ex}^{22} &\leq \Sigma_a \Sigma_t V_{sk}^{22} \end{aligned} \quad (\text{dissolution mode}) \quad (6)$$

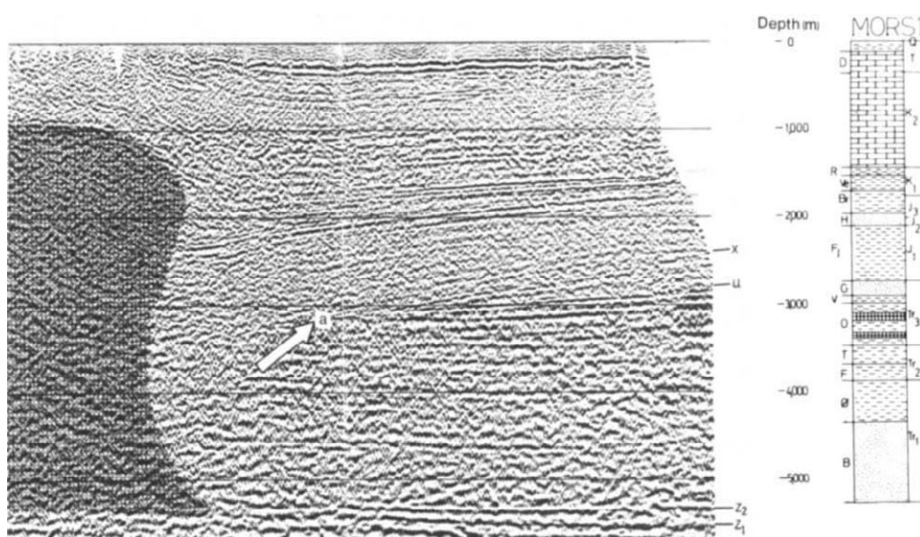
Many diapirs are characterized by significant overhangs. In the Trusheim model¹, overhang formation is supposed to occur by spreading of the diapir top. Alternatively, its formation can be thought of as a consequence of source layer depletion leading to 'necking' (Fig. 3c, right)¹⁰. Finally, overhang formation may be the result of combined necking and top spreading (Fig. 3c, left). If so, the positive and negative displacement volumes are vertically superposed and the surface sink will be less than the subsurface displacement volume. If necking is the dominant process, a surface sink will develop. To the 'overhang mode' of diapirism the following relation applies

$$0 \leq \Sigma_a \Sigma_t V_{sk}^{23} = \Sigma_a \Sigma_t V_{ex}^{23} \leq \Sigma_a \Sigma_t V_{sdd}^{23} \quad (\text{overhang mode}) \quad (7)$$

Significant lateral movements during overhang formation must be accommodated by deformation of pre-existing rim synclines. The strain ellipses (Fig. 3c) illustrate, in a general way, the state of finite strain imposed on the rim syncline by the overhang formation. Consequently, accurate volume estimation for salt diapirs with significant overhangs requires identification of the mechanism of overhang formation.

Let us consider a diapir (say that of Fig. 3a) and apply Trusheim's volume rule to its entire development. His rule asserts that the total excess volume is equal to the amount of finitely

Fig. 5 Migrated and depth-converted seismic reflection profile 7908 (Fig. 4). Acquisition and data processing by Prakla Seismos. The horizontal layering below the on-lap unconformity (au) signifies the pre-pillow series on top of a depleted source layer. Reflectors z_1 and z_2 probably originate from carbonatic and sulphatic source layer remnants. The line 'au' marks an on-lap and truncation structure which is interpreted as marking the onset of source layer depletion (see Fig. 2b). The Mors well, terminated at a depth of 5,303 m below sea level, is located close to the northeastern termination of line 7908. Stratigraphy and lithology from refs 20–23. Lithology: dots, sandstone; bars, shale; dots and bars, sandy shale; bricks, chalk. Cross-hatched, evaporites. Litho-stratigraphy: Bunter Formation (B); Ørsløv Formation (Ø); Falster Formation (F); Tønder Formation (T); Oddeund Formation (O); Vinding Formation (V); Gassum Formation (G); Fjerritslev Formation (Fj); Haldager Formation (H); Bream Formation (Br); Vedsted Formation (Ve); Røddby Formation (R). Chronostratigraphy: T₁, T₂, T₃, Lower, Middle and Upper Triassic, respectively; J₁, J₂, J₃, Lower, Middle and Upper Jurassic, respectively; K₁, K₂, Lower and Upper Cretaceous, respectively; D, Danian. T, Tertiary. Q, Quaternary.



displaced salt (V_{st} , Fig. 3a). Following the volume estimation of this work, however, the total excess volume is

$$V_{st}^1 + \Sigma_a \Sigma_i V_{so}^1 + V_{st}^2 \geq \Sigma_a \Sigma_i V_{ex}^{1+2} \geq V_{st}^1 + V_{st}^2 \quad (8)$$

in which $V_{st}^1 + V_{st}^2$ is seen to exceed V_{st} by an amount equal to the volume defined by the original source layer surface, the pillow surface and the diapir surface. Terming this volume the 'transition overlap volume' (V_{to}), equation (8) becomes

$$V_{st} + \Sigma_a \Sigma_i V_{so}^1 + V_{to} \geq \Sigma_a \Sigma_i V_{ex}^{1+2} \geq V_{st} + V_{to} \quad (9)$$

The excess volume is always larger than the volume of the salt which moved into the structure, so that Trusheim's volume rule, applied to a diapir, is incorrect, and only correct in the limit $v_{sed} > v_{up}$ when applied to a pillow structure (see equation (3)).

To obtain the excess volume as estimated above, it is necessary to correct the 'as measured' volumes ('am') for compaction: a correction due to the post-depositional compaction (post-correction, post(cc)) and a correction due to the syn-depositional compaction of the underlying sediments (pre-correction, pre(cc)). Post-depositional compaction leads to a positive post-correction equal in magnitude to the expansion of the unit in response to decompaction. Part of a unit under consideration is due to compaction of the underlying sediments, leading to a negative pre-correction. Then the compaction-corrected rim syncline volume ($V_{rs}(cc)$) is

$$V_{rs}(cc) = V_{rs}(am) + \text{post}(cc) - \text{pre}(cc) + \text{er}(c) \quad (10)$$

including an erosional correction ($\text{er}(c)$). The excess volume, in its corrected form, is

$$V_{ex} = V_{rs}(cc) - V_{rn}(cc) \quad (11)$$

where $V_{rn}(cc)$ is the regional normal corrected by a procedure similar to that of equation (10). Depending on lithology and position within the sequence, the total correction may be negative, positive or zero. The correction to any interval including the surface will be negative if erosional effects are negligible.

Mors post-diapirism

From uncorrected rim synclines^{1,5,7} it has been inferred that diapiric rates of $0.1\text{--}0.5 \text{ mm y}^{-1}$ are typically followed by rise rates at least one magnitude less^{5,7}. Trusheim¹ designated this 'slow tail' to diapirism as the post-diapiric phase, and its associated rim syncline as the tertiary rim syncline (see Fig. 1). Taking compactional effects into consideration, the question arises as to whether the excess volume of the post-diapiric rim syncline reflects salt structuring or whether it results from differential compaction.

The Mors salt diapir (Fig. 4) developed from a Zechstein source layer of unknown thickness (z_0). The profile to be discussed is derived from the seismic reflection profile 7908 (Fig. 5) which connects the Mors diapir with the deep well, Mors 1. The regional geology implies that the post-Oligocene history of the Mors area has been one of net erosion. It is therefore necessary to consider the effects of compaction on the 'post-diapiric' history, allowing for a non-vanishing erosion correction in equation (10). The decompaction calculations have been performed according to ref. 11. Maximum post-Oligocene erosion could have been $\sim 300 \text{ m}$. Removal of the Upper Cretaceous (UC) section leads to an 'expansion' of the pre-UC section of Mors 1 of 596 m assuming zero post-Oligocene erosion and 676 m assuming an erosion of 300 m. The corresponding expansions of the section through the secondary rim syncline (Fig. 6) are 679 and 749 m, respectively. The difference in expansion between the primary (Mors 1) and secondary rim synclines (83 m and 73 m, respectively) is not very sensitive to variations in the erosion correction of the relevant magnitude. This difference in expansion constitutes the negative pre-correction to the 'as measured' excess thickness. The latter has a maximum magnitude of 200–250 m, so that the compaction correction does not eliminate the 'as measured' excess thickness

of the post-LC rim syncline. Further calculations indicate that excess thicknesses persist through the entire UC and Danian chalk intervals, so that Mors did undergo extended, slow post-diapirism.

Neglecting compactional effects, the post-burial history of diapirs has been inferred in several studies^{7,12} on the basis of

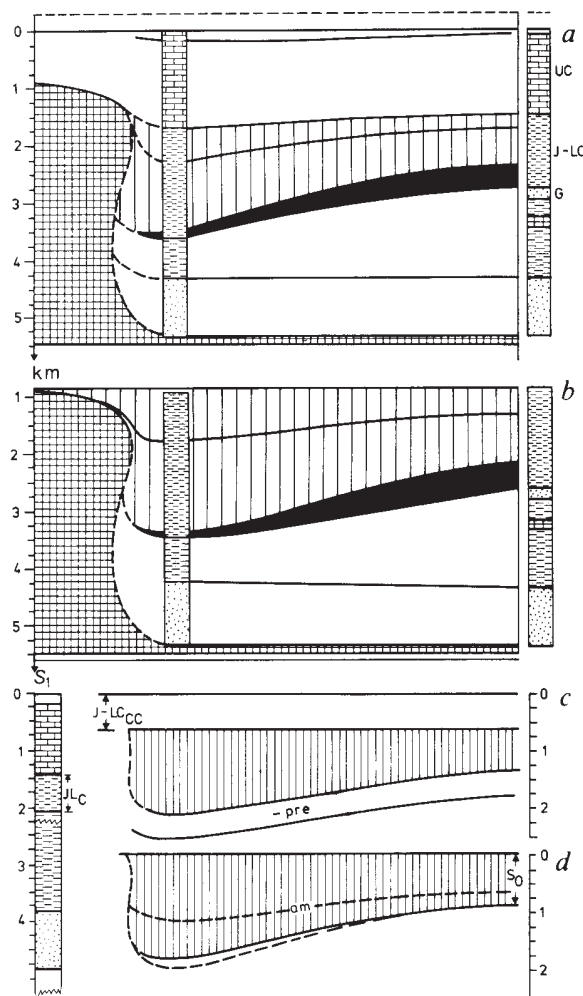


Fig. 6 Depletion history along 7908 and the total excess rim syncline. **a**, Present configuration. Black, the primary rim syncline. The lower boundary to the primary rim syncline corresponds to line au of Fig. 5. Vertical ruling, the secondary rim syncline. The lower boundary to the secondary rim syncline corresponds to reflector x of Fig. 5. White above ruling, the tertiary rim syncline. UC, Upper Cretaceous. J-LC, Jurassic and Lower Cretaceous. G, Gassum Formation. The upper stippled line indicates the maximum possible post-Oligocene erosion. Lithology symbols as in Fig. 5. **b**, End Lower Cretaceous configuration. Decompaction has been performed assuming an exponential decay of porosity (f) with depth (z), $f = f_0 \exp(-cz)$, where $1/c$ is the depth constant, the depth at which the porosity has decreased to $1/e$ of its original value), and the following material parameters¹¹: sandstone ($c = 0.27$; $f_0 = 0.49$), sandy shale ($c = 0.39$; $f_0 = 0.56$), shale ($c = 0.51$; $f_0 = 0.63$). Measuring z in km, c is in units of km^{-1} . **c**, Primary and secondary rim syncline (vertical ruling) after subtraction of a compaction-corrected Jurassic-Lower Cretaceous thickness (J-LC_{cc}) derived from J-LC of Skive 1 (S1) and after subtraction of the pre-correction (-pre). Skive 1 was terminated at a depth of 2,300 m below sea level; the Skive 1 column below this level was derived by a depth conversion of reflection data¹⁴. **d**, The compaction-corrected excess rim syncline (between zero level and the lower stippled line). Vertical ruling: the pre-UC part of the excess rim syncline. The excess thickness derived from uncorrected data: between zero level and am. All depth scales in km below sea level.

thickness differences between layers above and outside the diapir. The very large expansion of the pre-UC sections after removal of the post-Lower Cretaceous (LC) section at Mors shows that neglect of compactional effects in this procedure will lead to completely false conclusions as to post-burial rise rates. At Mors, this procedure¹³ would indicate a post-LC rise of 700 m, to which should be added an unknown height equivalent of dissolved salt. The excess volume shows that the true post-LC rise of the diapir at a maximum (with no dissolution) could have been ~100 m.

Mors total excess volume

The volume estimation leading to equation (9) will now be applied to profile 7908 in order to derive the total excess volume along it. The reflection profile (Fig. 5) shows a pronounced on-lap sequence of Gassum and lower Fjerritslev formation times. This angular unconformity ('au', Fig. 5) suggests fast pillow growth, similar to the model of Fig. 2b. There are only slight indications of pre-Gassum time salt depletion along 7908, so that au can be taken as the lower boundary to the primary rim syncline (Fig. 6a). In the preceding discussion of volume, the choice of a pillow phase growth model (Fig. 2a) was 'justified' by analogy with experimental work⁸. The on-lap sequence above the angular unconformity au (Fig. 5) shows that this choice is realistic in the case of Mors. The occurrence of similar on-lap structures adjacent to other salt structures in the Danish basin suggests a wider applicability of this pillow growth model.

Reflector x of Fig. 5 has been chosen to represent the pillow to diapir transition of Mors. When allowing for compactional effects, an abrupt change to excess thicknesses adjacent to the diapir takes place at approximately this level (corresponding to mid-Lower Jurassic time). The post-diapiric, diapiric, pillow and pre-pillow phases of the basin north of Mors can now be delineated (Fig. 6a), acknowledging that the diapiric to post-diapiric transition is arbitrarily positioned at the LC-UC transition.

The geometry of the 'total' rim syncline (encompassing the total structure development), except for minor post-LC movements, is shown in its positively post-corrected form in Fig. 6b. The negative pre-correction is calculated by decompaction to the lower boundary of the total rim syncline, giving the corrected, total rim syncline (Fig. 6c). The total excess volume can now be derived by subtraction of the regional normal. The original source layer thickness, s_0 , will then appear as the minimum height of the excess rim syncline. The crucial point of this process is the derivation of the 'regional normal', because this thickness exists nowhere near Mors. The choice of the 'regional normal' will be explained below.

A comparison of seismic sections over the Rødding Flat (Fig. 4) and sections crossing the Skive 1 well and the Skive structure indicates that the J-LC of Skive 1 is similar to the J-LC of the Rødding Flat west of the Skive pillow structure where no J-LC salt movement occurred¹⁴. The J-LC thickness in Skive, being 649 m, can therefore be used as the 'regional normal'. To find the total excess thickness, the compaction-corrected regional normal (J-LC_{cc}, Fig. 6c) is subtracted from the 'top' of the total

rim syncline, while the negative pre-correction is subtracted from the 'bottom'. Subsequently, the remaining part of the rim syncline (Fig. 6c, ruled) is decompacted to give the compaction-corrected excess thickness (Fig. 6d, ruled). The lower stippled line of Fig. 6d shows the thickness to be added due to post-diapiric movements. This gives a source layer depletion of 850 ± 50 m (s_0 of Fig. 6d). Adding to this the remaining Zechstein thickness (probably a basal carbonatic and sulphatic facies, 250 ± 50 m thick) yields an original Zechstein thickness (z_0) of $1,100 \pm 100$ m.

The total excess volume is clearly as much as 50% larger than the total salt volume (allowing for three-dimensional effects¹⁵), illustrating the significance of the overlap volumes (equation (9) and Figs 2a, 3a) in the volume estimation. Following Trusheim, this would lead to erroneous conclusions about the amount of dissolved salt. It is also seen (Fig. 6d) that the true excess volume is considerably larger (again ~50%) than the 'as measured' excess volume.

Conclusions and implications

The discussion of volume estimation for salt diapirism leads to the conclusion that the total excess volume cannot be simply related to the amount of salt as claimed by Trusheim's¹ volume rule. It is necessary to account for the interrelation of several volume terms—surface sink and source volumes, subsurface displacement volumes and overlap volumes—and to relate these to the excess volume, taking into account the fact that the latter can vary with varying rates of regional sedimentation.

For the discussion of the disposal of high-level waste in salt structures, it is essential to have maximum confidence in both growth histories and growth rates. The example of Mors shows that this goal can only be reached by the use of rigorous volume estimation, for without it, diapiric rise rates may be considerably underestimated (Fig. 6d). The discussion of the post-burial history of Mors shows, on the other hand, that it is possible to document very long periods of vanishingly small post-diapiric movement, a period whose growth rate in previous studies has been grossly overestimated due to the neglect of compactional effects.

The discussion of volume estimation is of relevance to another applied aspect of salt structures, petroleum exploration. In this context, sink and source volume relations should be emphasized. In the 'dissolution mode' of diapiric movement the diapir itself does not provide a source to fill the sink volume. In this mode, accumulation of source material for petroleum generation may be enhanced in the sink, in contrast to the diapiric periods without dissolution.

Finally, to perform trustworthy analyses of tectonic subsidence histories of sedimentary basins containing salt structures, it is essential to allow for salt-induced thickness variations. In recent studies^{11,16,17} of North Sea subsidence, salt-induced sedimentation has been neglected, despite the fact that many exploration wells have been located on strongly salt-influenced structures. This aspect is exemplified in ref. 18.

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Direct evidence for chromosomal inversion during T-cell receptor β -gene rearrangements

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A germline T-cell receptor variable region (V_β) gene segment ($V_\beta 14$) has been mapped 10 kilobases to the 3' side of the constant region ($C_\beta 2$) gene. The $V_\beta 14$ gene segment is in an inverted transcriptional polarity relative to the diversity-region (D_β) and joining-region (J_β) gene segments and the C_β genes. Analyses of a T-cell clone (J6.19), which has productively rearranged the $V_\beta 14$ gene segment, indicate that the productive V_β - D_β - J_β rearrangement and its reciprocal flank recombination product are linked and located at either border of a chromosomal inversion. These data demonstrate for the first time a linkage between mammalian V and C genes and verify that a functional T-cell receptor V_β gene can be constructed through a chromosomal inversion.

T-CELL antigen receptors are disulphide-linked heterodimers constituted of two chains designated α and β (refs 1-3), each divided into variable (V) and constant (C) regions⁴. The T-cell receptor V_β region is encoded by three distinct sets of DNA segments designated variable (V_β), diversity (D_β) and joining (J_β)⁵⁻⁹ which join during T-cell differentiation to generate a complete V_β gene⁵. The β -gene family contains two closely linked constant-region (C_β) genes denoted $C_\beta 1$ and $C_\beta 2$, each of which is preceded by a cluster of six functional joining-region (J_β) gene segments⁵⁻⁷, at least one diversity-region (D_β) gene segment^{8,9} and an unknown number of V_β gene segments.

Before somatic rearrangements, the 3' side of the V_β gene segments, the 5' side of the J_β gene segments and both sides of the D_β gene segments are flanked by recognition sequences for DNA rearrangement. These recombination signal sequences are comprised of three components—a conserved heptamer, a non-conserved spacer sequence of 12 or 23 nucleotides and a conserved A+T-rich nonamer⁵⁻⁹. As in immunoglobulin genes, DNA rearrangement occurs only when one gene segment has a 12-base-pair (bp) recognition sequence and the second has a 23-nucleotide signal sequence.

Several mechanisms have been suggested for the joining process^{10,11}. First, the DNA between the two gene segments may loop out and be deleted by virtue of a site-specific recombination event joining the two gene segments (looping out-excision model)^{12,13}. Second, during mitotic duplication of a chromosome, an unequal crossing-over may occur between the 3' end of a gene segment on one sister chromatid and the 5' end of the second gene segment on the second sister chromatid. The two resulting chromatids would be segregated at the next cell division, giving rise to two types of daughter cells. One cell would contain the joined gene segments; this cell and its progeny would appear to have deleted all the DNA between the recombined gene segments. The second cell would contain a reciprocal flank recombination product with the two recombination signals fused back-to-back at their coding-proximal borders. This model is denoted unequal sister chromatid exchange^{14,15}. A third model, particularly relevant to the data presented in this article, requires that the two gene segments to be joined are oriented in opposite transcriptional polarity in the germline DNA. Inversion of the stretch of chromosomal DNA between the 3' (or the 5') ends of these two gene segments would result in the formation of (1) an appropriate coding joint with both gene segments in the same transcriptional direction and (2) a reciprocal flank recombination joint. This inversion model does not lead to the

loss or gain of intervening sequences between the joined gene segments but merely to their rearrangement¹⁵⁻¹⁸. Finally, the looping out-excision model may have an alternative form in which the excised loop is reintegrated back into the genomic (looping out-excision-reintegration model)^{19,20}. However, since recombination events may occur in several steps during ontogeny^{17,21-24}, a combination of inversion or sister chromatid

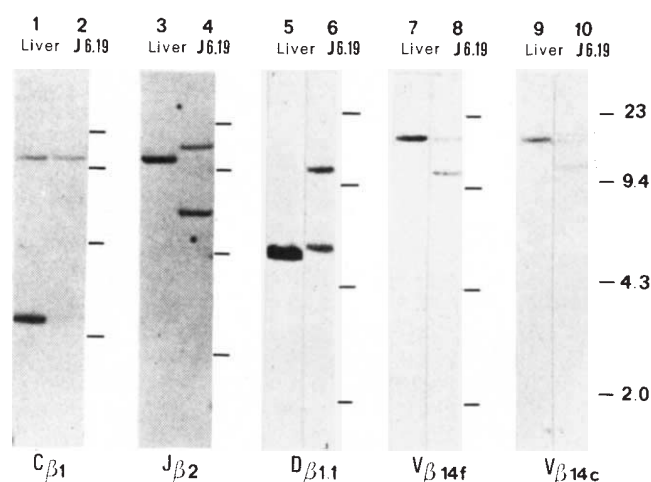
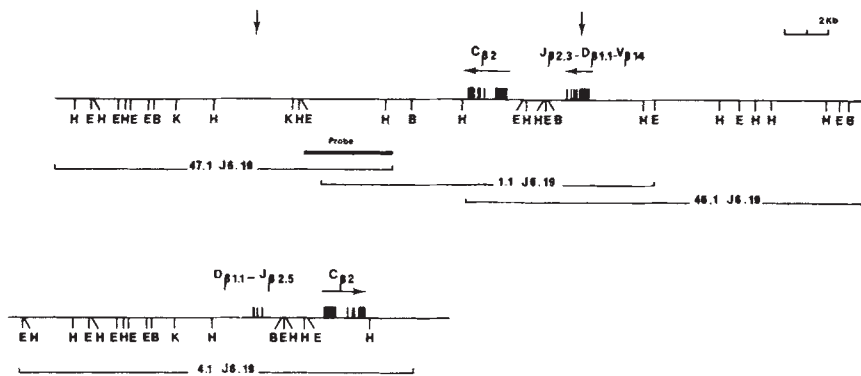


Fig. 1 Southern blot analyses of J6.19 T-cell DNA. DNAs from B10.A liver and J6.19 T cells were digested to completion with *Eco*RI (lanes 1, 2) or *Bam*HI (lanes 3-10) and after gel electrophoresis and transfer, hybridized to ³²P-labelled single copy probes. Probes were isolated from either the germline β -chain gene cosmid 2.3W7 (ref. 6) or the λ genomic clones 47.1 J6.19 and 46.1 J6.19 (this paper). The $C_\beta 1$ probe is a 2.5-kb *Eco*RI fragment that hybridizes to $C_\beta 1$ as well as to $C_\beta 2$ sequences because of homology between these two genes. Probe $J_\beta 2$ is a 2.3-kb *Eco*RI fragment which contains all of the $J_\beta 2$ gene segments. Probe $D_\beta 1.1$ is a 3.2-kb *Hind*III-*Bam*HI fragment which detects sequences 5' to $D_\beta 1.1$, the $D_\beta 1.1$ and the two 5' $J_\beta 1$ gene segments. Two probes were used to analyse the $V_\beta 14$ gene segment. The first one, denoted $V_\beta 14c$, corresponds to a 250-bp *Mbo*II fragment contained entirely within the $V_\beta 14$ coding sequence. The second probe, denoted $V_\beta 14f$, is a 4-kb *Eco*RI-*Sal*I fragment corresponding to the non-coding 3' side of the $V_\beta 14$ gene segment. The parental B10.A strain liver DNA is included in each analysis for comparison of the rearrangements with the germline pattern. Size markers are indicated in kb.

Fig. 2 Restriction maps of the genomic clones containing the β -gene rearrangements observed in J6.19 T lymphocytes. Exons encoding V_β , D_β and J_β gene segments and C_β genes are indicated by vertical lines or boxes above the horizontal line. Cleavage sites are indicated by vertical lines below the horizontal line. Restriction enzyme abbreviations are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I. A genomic library was constructed from *Sau*3A partially digested J6.19 DNA using the λ bacteriophage vector EMBL-3 (ref. 41). Screening of the library was carried out according to Maniatis *et al.*⁴² using probes specific for D_β 1.1, C_β 1, J_β 2 and C_β 2 sequences (see below). DNA was prepared from the isolated genomic clones and mapped with the restriction enzymes indicated. The location of the V_β , D_β , J_β and C_β sequences was determined by Southern blot analyses of restriction fragments from the genomic clones. Some hybridization probes have been described previously; they correspond either to subclones made from the germline β cosmid 2.3W7^{6,11} or to oligonucleotides specific (1) for each of the 12 functional J_β gene segments, (2) for the 5'-flanking sequences of the D_β 1.1 and D_β 2.1 gene segments and (3) for the D_β 1.1 and D_β 2.1 gene segments^{27,35}. The restriction fragments contained in these clones account for all the rearrangements detected in the Southern blot analysis of KB5-C20 DNA (see Fig. 1). The position of the 4-kb *Eco*RI-*Sal*I fragment isolated from clone 47.1 J6.19 and used to probe the J6.19 genomic library is indicated by a filled box. Vertical arrows indicate the border of the chromosomal inversion. Horizontal arrows indicate 5' to 3' orientation.



exchange and deletion can explain all existing observations^{11,17,25}.

We report here the molecular linkage of a germline T-cell receptor V_β gene segment 3' to the C_β locus. This V_β gene segment (V_β 14) is oriented in opposite transcriptional polarity relative to the D_β and J_β gene segments and the C_β genes. Analyses by chromosomal walking techniques of a T-cell clone that has productively rearranged the V_β 14 gene segment demonstrate that the productive V_β - D_β - J_β rearrangement and its reciprocal flank recombination product are linked on the same piece of DNA and located at either border of a chromosomal inversion. Taken together, these data represent to our knowledge the first formal demonstration of a chromosomal inversion mechanism operating during V_β to D_β - J_β joining.

β -Gene rearrangements in T cell J6.19

J6.19 is a proliferative T-cell clone that originates from a B10.A mouse and responds to hen egg ovalbumin presented by I-A^k-bearing cells²⁶. DNA from J6.19 cells was digested with *Eco*RI and *Bam*HI and Southern blots of the digested DNA were then hybridized with the probes described in Fig. 1. Hybridization of the C_β 1 probe to *Eco*RI-digested B10.A liver DNA shows the C_β 1 (lower band) and C_β 2 (upper band) genes in their germline configurations. *Eco*RI digests of J6.19 DNA hybridized to the C_β 1 probe demonstrate that both C_β 1 genes are deleted and that at least one C_β 2 gene is present. Hybridization with the J_β 2 probe shows that the J_β 2 cluster is rearranged on both chromosomes of J6.19 T cells. Finally, hybridization with the D_β 1.1- J_β 1.1- J_β 1.2 probe indicates that this region is twice rearranged in J6.19 cells.

To further delineate the nature of the β -gene rearrangements in J6.19 T cells, we have constructed a complete λ phase genomic library and isolated the clones encompassing all of the β -gene rearrangements observed during Southern blot analyses. Three classes of clones were found for J6.19 DNA, represented by clones 4.1, 46.1 and 47.1 (Fig. 2). The localization within the clones of the D_β and J_β gene segments and the C_β genes was determined using the hybridization probes described in Fig. 2 legend. Clone 46.1 has no C_β 1 gene. Oligonucleotide probes show that the D_β 1.1 gene segment is joined to the J_β 2.3 gene segment and that the 5'-flanking sequence for the D_β 1.1 gene segment is absent—therefore, this clone contains a V_β - D_β 1.1- J_β 2.3 rearrangement. Clone 4.1 has a D_β 1.1 gene segment joint to a J_β 2.5 gene segment and does contain 5'-flanking sequence for the D_β 1.1 gene segment—therefore, this clone encodes a partial D_β 1.1- J_β 2.5 rearrangement. Clone 47.1 is novel in that

it contains the rearranged 5'-flanking D_β 1.1 sequence and does not hybridize to the J_β or C_β probes. A vertical arrow indicates the localization of the 5'-flanking D_β 1.1 sequence inside clone 47.1 (Fig. 2). This clone will be discussed later as it has important implications for β -gene rearrangement mechanisms. These three β -gene rearrangements represent all of those observed by Southern blot analyses in the J6.19 T cells (Fig. 1).

The complete variable gene and novel rearrangements have been subcloned and sequenced (Fig. 3). The V_β rearrangement in λ clone 46.1 appears functional in that it has appropriate leader and V_β gene segment sequences and a V_β - D_β - J_β joining that maintains the proper translational reading frame (Fig. 3). The sequence of the V_β gene segment from clone 46.1 of the J6.19 cells is reported here for the first time. This V_β segment detects a single hybridizing band when used to probe a Southern blot of B10.1 liver DNA (Fig. 1) and, accordingly, defines a new single-member V_β subfamily. According to the nomenclature introduced by Barth *et al.*²⁷ and Kronenberg *et al.*¹⁰, this V_β gene segment will be denoted V_β 14.

The novel rearrangement observed in clone 47.1 corresponds to a flank recombination product with the heptamer sequences of V_β and D_β rearrangement signals joined in a back-to-back orientation (Fig. 3). Similar rearrangements have been reported for some immunoglobulin genes¹⁴⁻²⁰. Does the flank recombination product of clone 47.1 correspond to the reciprocal of the V_β - D_β rearrangement defined by clone 46.1? If so, 5' D_β 1.1 recognition sequences for DNA rearrangement should be joined to the 3' V_β 14 recognition sequences. To answer this question we have isolated (see below) and determined the nucleotide sequence of the germline V_β 14 gene segment (Fig. 3). Comparison of the coding sequences of the germline V_β 14 gene segment and its rearranged counterpart found in J6.19 T cells demonstrates no nucleotide differences and further illustrates the lack of somatic hypermutation in T-cell receptor β genes. Moreover, comparison of the 3'-flanking sequence of the germline V_β 14 gene segment and of the V_β recognition sequence of the V_β - D_β flank recombination product found in J6.19 T cells demonstrates a complete nucleotide identity (Fig. 3). Therefore, these data indicate that the V_β 14 gene segment rearranged in J6.19 T cells has not segregated away from its reciprocal flank recombination product. Since the sister chromatid exchange model requires that the reciprocal events segregate independently in the two daughter cells, the β -gene rearrangement data obtained with J6.19 T cells are only consistent with either an inversion model or with the looping out-excision-reintegration model.

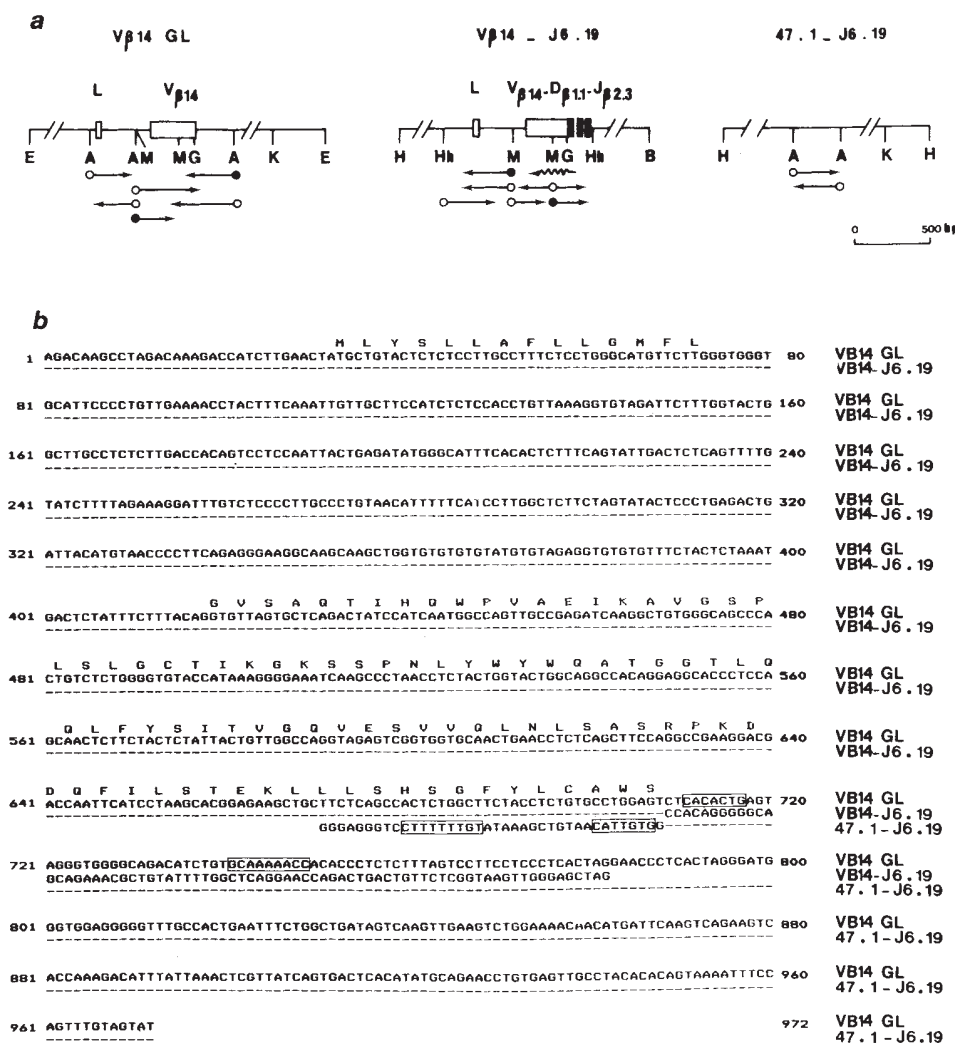


Fig. 3 Sequencing strategies (**a**) and sequences (**b**) of the V β 14 germline segment, the V β 14-J β 6.19 coding joint and the 47.1-J β 6.19 reciprocal flanking joint. The sequence of the V β 14 germline segment denoted VB14 GL was obtained from a 5-kb *Eco*RI fragment derived from a cosmid 40.1W7 (see Fig. 4). The V β 14-J β 6.19 sequence corresponds to the rearranged V β 14 gene segment found in J β 6.19 T cells. The sequence denoted 47.1-J β 6.19 corresponds to the V β 14-D β 1.1 flank recombination product found in J β 6.19 T cells. This latter sequence was obtained from a 300-bp *Acc*I fragment hybridizing to a D β 1.1 flanking oligonucleotide probe. **a**, The sequences were obtained by either the dideoxy-chain termination methods (wavy arrows) or by the procedure of Maxam and Gilbert. Closed circles indicate a 5' end labelling and open circles a 3' end labelling. The following abbreviations are used: L, leader; V β , variable gene segment. The restriction enzyme abbreviations are as follows: A, *Acc*I; E, *Eco*RI; G, *Bgl*I; H, *Hind*III; Hh, *Hha*I; K, *Kpn*I; M, *Mbo*II. **b**, Comparison of the V β 14 GL nucleotide sequence with the V β 14-J β 6.19 and 47.1-J β 6.19 sequences. The predicted amino-acid sequence encoded by the V β 14 exons is indicated above using the one-letter amino-acid code. Nucleotide identities with the upper line are shown with dashes. The D β and V β recognition sequences have been boxed. Note that the 3' side of the germline V β 14 gene segment is flanked by the sequence CAACTG-(23 nucleotides)-GCAAAAACC which is similar to the recognition signals found 3' to the immunoglobulin V region genes¹⁰.

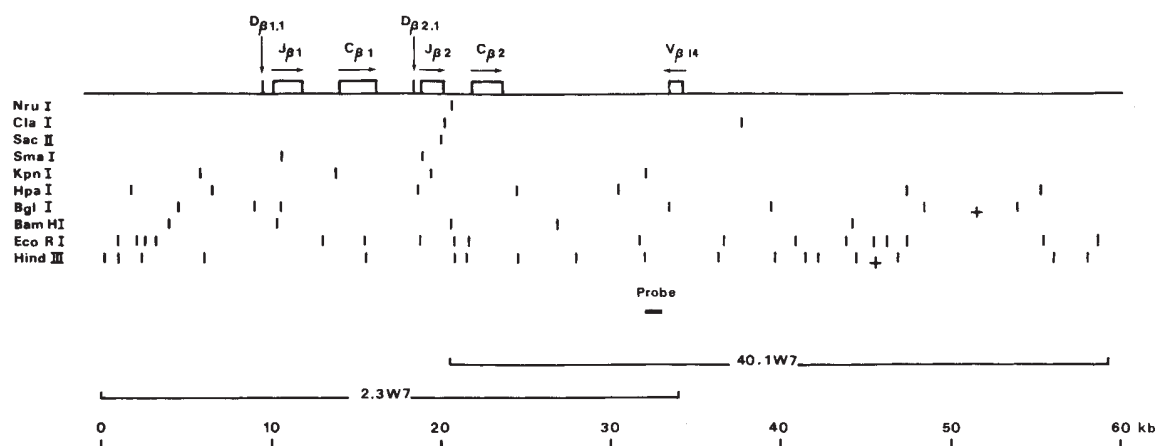


Fig. 4 Restriction map of the clones 2.3W7 and 40.1W7 isolated from a cosmid library constructed with DNA from the livers of B10.WR7 mice. Boxes or vertical lines above the horizontal line indicate the exons encoding V β , D β , J β and C β sequences. The cosmid clone 2.3W7 has been described previously⁶. The position of the 900-bp *Kpn*I-*Sau*3A probe used to isolate the cosmid 40.1W7 is indicated by a filled box. The cosmid clone 40.1W7 was mapped with the restriction enzymes indicated by single and double digestions. A (+) between two restriction sites indicates the presence of unassigned sites in this region for the same enzyme. The orientation and the location of the different gene segments were determined by Southern blot analyses of restriction fragments. The hybridization probes are described in the legend to Fig. 2. Arrows indicate 5' to 3' transcriptional orientation.

$V_{\beta}14$ gene segment lies 10 kb 3' to $C_{\beta}2$

Further clues concerning the location of the $V_{\beta}14$ gene segment were unexpectedly obtained during analysis of the KB5-C20 alloreactive cytotoxic T-cell clone generated from a B10.BR mouse and directed against the H-2K^b molecule²⁸. KB5-C20 T cells present two incomplete D_{β} - J_{β} rearrangements on one chromosome and a complete V_{β} - D_{β} - J_{β} rearrangement on the second chromosome (data not shown). In order to determine whether the V_{β} gene segment used in KB5-C20 T cells had been reported previously, the λ genomic clone corresponding to a complete V_{β} - D_{β} - J_{β} rearrangement (clone 28.1 KB5) was hybridized with a panel of probes specific for various V_{β} gene segments. Unexpectedly, clone 28.1 KB5 hybridized with two different V_{β} probes corresponding to V_{β} (refs 27, 29) and $V_{\beta}14$ (this paper). These two V_{β} gene segments show less than 20% homology. We have subsequently determined the relative positions of the $V_{\beta}2$ and $V_{\beta}14$ gene segments within the 28.1 KB5 clone. The $V_{\beta}2$ gene segment mapped to the 5' side of the $J_{\beta}2$ - $C_{\beta}2$ sequences. Surprisingly, the $V_{\beta}14$ gene segment mapped about 10 kilobases (kb) to the 3' side of the $C_{\beta}2$ gene. However, T lymphocytes undergo complex β -gene rearrangements during T-cell differentiation¹¹ and these may have brought the $V_{\beta}14$ gene segment to its position in clone 28.1 KB5. We have therefore analysed the germline localization of the $V_{\beta}14$ gene segment before any β -gene rearrangement.

We have described previously a 34-kb mouse cosmid clone denoted 2.3W7 containing the $C_{\beta}1$ and $C_{\beta}2$ genes with their associated D_{β} and J_{β} gene segments⁶. This clone was isolated from a cosmid genomic library constructed from B10.WR7 liver DNA. We have now extended the molecular map to the 3' side of the $C_{\beta}2$ gene by chromosomal walking. The single-copy probe used for that purpose corresponds to a 900-bp *KpnI*-*Sau3A* fragment derived from the 3' end of the cosmid clone 2.3W7 (Fig. 4). This procedure yielded the cosmid clone 401.1 W7 and allowed us to extend our map about 35 kb to the 3' side of the $C_{\beta}2$ gene (Fig. 4). Southern blots of the 401.1 W7 cosmid clone were then hybridized with the $V_{\beta}14$ gene segment probe. This probe was found to hybridize strongly to a 5-kb *EcoRI* fragment located to the 3' side of the $C_{\beta}2$ gene. Direct confirmation that this 5-kb *EcoRI* fragment does indeed encode the $V_{\beta}14$ gene segment was provided by DNA sequence analysis (see above). Interestingly, the germline $V_{\beta}14$ gene segment has a 5' to 3' transcriptional orientation that is opposite to that found for the D_{β} and J_{β} gene segments and the C_{β} genes (Fig. 4). Thus, these results demonstrate that in germline DNA the $V_{\beta}14$ gene segment lies 10 kb to the 3' side of the $C_{\beta}2$ gene and is oriented in opposite transcriptional polarity relative to the D_{β} and J_{β} gene segments and the C_{β} genes.

$V_{\beta}14$ rearrangement by chromosomal inversion

The $V_{\beta}14$ gene segment constitutes to our knowledge the first example in which a germline V_{β} segment has been linked to the locus encoding the D_{β} and J_{β} gene segments and the C_{β} genes. Therefore, further analysis of the DNA rearrangements accompanying the productive $V_{\beta}14$ joining event observed in J6.19 T cells has provided additional insights into the mechanism of V_{β} to D_{β} - J_{β} joining. Because the germline $V_{\beta}14$ segment is oriented in an opposite polarity relative to the D_{β} and J_{β} gene segments and the C_{β} genes, one way to interpret the reciprocal recombination products found in J6.19 T cells involves a chromosomal inversion event. As outlined in Fig. 5, if the $V_{\beta}14$ gene segment joins to the $D_{\beta}1.1$ - $J_{\beta}2.3$ segment by chromosomal inversion, the reciprocal flank recombination product would be formed and retained on the same chromosome bearing the productive $V_{\beta}14$ - D_{β} - J_{β} rearrangement. Furthermore, because we have shown that the $V_{\beta}14$ gene segment is located about 10 kb to the 3' side of the $C_{\beta}2$ gene, the reciprocal flank recombination product should be found about 10 kb to the 3' side of the $C_{\beta}2$ gene. To test this supposition, we attempted to link the genomic clone encompassing the $V_{\beta}14$ - $D_{\beta}1.1$ - $J_{\beta}2.3$ rearrange-

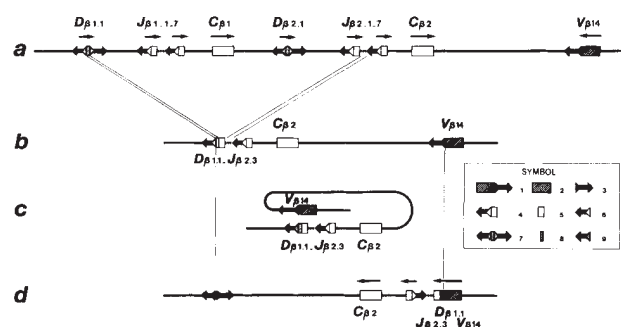


Fig. 5 Model explaining the presence in J6.19 T cells of the productive V_{β} - D_{β} - J_{β} rearrangement and of its reciprocal recombination product at either border of a chromosomal inversion. The chromosomal structures depicted in *a* and *d* are based on the data presented in Figs 4 and 2, respectively. Intermediate structures generated during the proposed pathway of rearrangement are illustrated in *b* and *c*. *a*, Organization of the germline $C_{\beta}1$ and $C_{\beta}2$ genes with their associated D_{β} ($D_{\beta}1.1$ and $D_{\beta}1.2$) and J_{β} ($J_{\beta}1.1$ -7 and $J_{\beta}2.1$ -7) gene segments. The germline $V_{\beta}14$ segment lies 10 kb to the 3' side of the $C_{\beta}2$ gene and is oriented in opposite transcriptional polarity relative to the D_{β} , J_{β} and C_{β} sequences. Two stepwise recombinations must have occurred to form the final structure. *b*, An initial $D_{\beta}1.1$ - $J_{\beta}2.3$ rearrangement occurred on the chromosome. This scheme is consistent with recent data suggesting that D_{β} to J_{β} rearrangements occur prior to V_{β} to D_{β} - J_{β} rearrangements (ref. 24 and R. Haars, M. Kronenberg and L.H., in preparation). The absence of both $J_{\beta}1$ clusters and both $C_{\beta}1$ genes in J6.19 T cells supports the concept that D_{β} to J_{β} rearrangement occurs either by the looping out-excision or by the sister chromatid exchange model. *c*, The $V_{\beta}14$ segment is recombined with the $D_{\beta}1.1$ - $J_{\beta}2.3$ segment much as in the model of inverted joining hypothesized in refs 16, 18 and 25. During this process the $V_{\beta}14$ gene segment uses its 3' recognition sequence to mediate joining with the $D_{\beta}1.1$ segment. *d*, The resulting product would have kept the entire DNA stretch located between the recombination points. A productive $V_{\beta}14$ - $D_{\beta}1.1$ - $J_{\beta}2.3$ joining would be formed with segments in the same transcriptional polarity. The reciprocal flank recombination product is also formed and lies 10 kb to the 3' side of the $C_{\beta}2$ gene. The homologous chromosome is not shown; it presents a partial $D_{\beta}1.1$ - $J_{\beta}2.5$ rearrangement and a $V_{\beta}14$ gene segment in germline configuration (see text). Arrows indicate 5' to 3' transcriptional orientation. Distances are not drawn to scale. Symbol for gene segments are as follows: 1, germline $V_{\beta}14$ segment; 2, V_{β} coding sequences; 3, $V_{\beta}14$ joining signal; 4, germline J_{β} segment; 5, J_{β} coding sequences; 6, J_{β} joining signal; 7, germline D_{β} segment; 8, D_{β} coding sequences and 9, D_{β} joining signals.

ment (clone 46.1 J6.19, Fig. 2) to the V_{β} - $D_{\beta}1.1$ flank recombination product (clone 47.1 J6.19, Fig. 2). For that purpose we rescreened a complete λ genomic library made from J6.19 T cells using a single-copy probe derived from the genomic clone 47.1 J6.19 (Fig. 2) and isolated one clone, denoted 1.1 J6.19. Subsequent mapping with restriction enzymes and Southern blot analyses of restriction fragments established that the 1.1 J6.19 clone does indeed overlap with the 46.1 J6.19 and 47.1 J6.19 genomic clones (Fig. 2). Therefore, we conclude that the $V_{\beta}14$ - $D_{\beta}1.1$ - $J_{\beta}2.3$ rearrangement and its reciprocal flank recombination product are linked on the same piece of DNA and located at either border of a chromosomal inversion. It was possible to determine the extent of the inversion region by comparing the restriction map shown in Fig. 2 with the one corresponding to the germline configuration (Fig. 4). Moreover, as predicted by the inverted joining model depicted in Fig. 5, the reciprocal flank recombination product is indeed located 10 kb to the 3' side of the $C_{\beta}2$ gene. Integrity of the molecular map presented in Fig. 2 was confirmed by Southern blot analyses of J6.19 T-cell DNA cleaved with *Bam*HI and hybridized with four single-copy probes encompassing the inversion (Fig. 1). This analysis also indicates that the $V_{\beta}14$ segment located on the homologous chromosome lies in the germline configuration 10 kb to the 3'

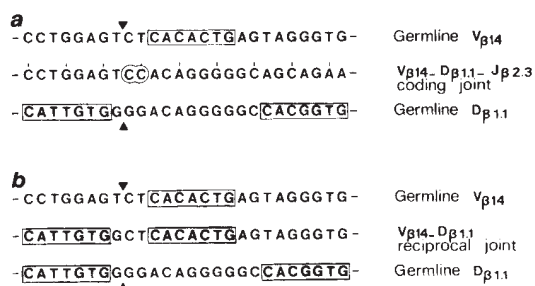


Fig. 6 Comparison of the sequences of the coding (*a*) and reciprocal (*b*) junctions located at either border of the J6.19 inversion. Heptameric elements of the joining signals are boxed; the spacer regions and nonamer elements of the joining signals are not included in the sequence shown. Nucleotide regions not accounted for by germline V_β or D_β sequences (*N* region) are enclosed in circles. The sites of crossing-over are shown by black triangles. The $V_\beta 14-D_\beta 1.1-J_\beta 2.3$ reading frame is indicated by ticks above the first base of each codon. The sequences corresponding to the $V_\beta 14-D_\beta 1.1-J_\beta 2.3$ coding joint, the $V_\beta 14-D_\beta 1.1$ reciprocal joint and the $V_\beta 14$ germline segments are from this article (Fig. 3). The germline $D_\beta 1.1$ segment has been reported previously^{9,28}.

end of the $C_{\beta}2$ gene. Taken together, these results demonstrate unequivocally that the $V_{\beta}14$ to $D_{\beta}-J_{\beta}$ rearrangement observed in J6.19 T cells has occurred through a 14-kb chromosomal inversion.

DNA joints not precisely recombined

The 14-kb inversion observed in J6.19 T cells creates two new DNA joints. One is the V_β - D_β coding joint and the other is the reciprocal flank recombination signal joint. Analysis of the sequence of the V_β 14- D_β 1.1 reciprocal joint indicates that three nucleotides separate the two heptamers (Fig. 6b). Two of these intervening nucleotides appear to be derived from germline sequences 5' to the V_β 14 heptamer whereas the third may originate from the 3' side of the D_β 1.1 heptamer. Thus, all three intervening bases could be accounted for by germline sequences. This observation suggests that the recognition sequences need not be precisely joined in the rearrangement process^{18,25}. Such an intervening triplet of nucleotides has not been found in V_κ to J_κ reciprocal joints. In fact, in each κ example reported to date, the two heptamers are joined precisely back to back^{15,18,19,23,24,30}. Unfortunately there is no way of knowing on the basis of a single example if the nucleotide triplet separating the two heptamers should be considered as a characteristic feature of the V_β - D_β reciprocal joint.

Analysis of the coding joint (Fig. 6a) indicates that two nucleotides have been deleted from the coding sequences of the $D_\beta 1.1$ gene segment. Because the germline $D_\beta 1.1$ gene segment sequence is derived from B10.A, the same strain from which J6.19 T-cell clone is derived, we may conclude that two nucleotides have been deleted from the coding sequences of the $D_\beta 1.1$ gene segment. Therefore, joining does not conserve all of the sequences present in the precursor elements. Furthermore, two bases between the joined $V_\beta 14$ and $D_\beta 1.1$ gene segments cannot be accounted for in either germline gene segment and may represent an N region. N regions consist of short stretches of random nucleotides appearing as insertions at $V-D$ and $D-J$ junctions of both immunoglobulin heavy-chain^{16,31-34} and T-cell receptor β genes^{8,9,35}. A direct conclusion obtained from the comparison of the reciprocal recombination products and of their precursor sequences is that DNA elements are asymmetrically joined during the $V_\beta-D_\beta$ recombination event. The reciprocal joint sequence arises entirely from the precursor germline sequences, whereas the coding joint sequence shows both an N region and a small number of base pairs missing relative to the precursor sequences. Similar conclusions have

been reached during the analysis of the products formed during V_κ to J_κ joining events^{18,25}.

Discussion

We have mapped the germline $V_{\beta}14$ gene segment 10 kb to the 3' side of the $C_{\beta}2$ gene and shown that it presents an inverted transcriptional polarity relative to the D_{β} and J_{β} gene segments and the C_{β} genes. Having examined a productive $V_{\beta}14$ joining event, we present evidence that V_{β} - D_{β} joining can occur via chromosomal inversion. These results have been obtained from genomic libraries constructed from B10.A, B10.BR or B10.WR7 DNA. These three strains of mice share the same chromosome 6 derived from the B10 background³⁶. To extend our results to other strains of laboratory mice, we have performed Southern blot analyses of AKR, BALB/c, C3H and C57BL/6 liver DNA cleaved with seven different restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I and *Pvu*II) and hybridized these DNAs with probes $C_{\beta}2$, $V_{\beta}14c$ and $V_{\beta}14f$ (see legend to Fig. 2). In all cases the sizes of the genomic fragments correspond with those predicted by the map obtained from B10.WR7 mice. Further support for this model was obtained by the direct analysis of a β -genomic clone derived from A.TL DNA (D.B. and C.D., unpublished observations).

An analysis of the β -gene rearrangements in the T J6.19 cells suggests they occurred in the following manner. Initial $D_{\beta}1.1$ - $J_{\beta}2$ rearrangements occurred on both β -locus chromosomes. This contention is consistent with recent data suggesting that D_{β} - J_{β} rearrangements occur before V_{β} to $D_{\beta}J_{\beta}$ rearrangements²⁴ (R. Haars, M. Kronenberg and L.H., in preparation). Since the $J_{\beta}1$ clusters and $C_{\beta}1$ gene have been deleted from both chromosomes in the J6.19 cells, the $D_{\beta}1$ - $J_{\beta}2$ joining events must have occurred by the looping out-excision or by the sister chromatid exchange mechanisms. The chromosome 6 represented by clone 4.1 presumably did not undergo any further β rearrangement (Fig. 2). The V_{β} to $D_{\beta}J_{\beta}$ rearrangement on the other chromosome 6 presumably led to the V_{β} - D_{β} flank recombination products of clone 47.1, thus requiring an inversive mechanism for DNA rearrangements (Fig. 5). Indeed, the inversion model would predict that each time the $V_{\beta}14$ gene segment of the J6.19 cells is used, there should be a flank recombinational product present in that T cell. Thus, T cell J6.19 must use two distinct mechanisms for DNA rearrangement—one for $D_{\beta}J_{\beta}$ joining and a second for V_{β} to D_{β} - J_{β} joining.

We have shown that the joining signals of $V_\beta 14$ and $D_\beta 1.1$ have recombined, leading to an inversion of a region of the T-cell receptor β locus. Such a V_β - D_β joining could not have occurred by unequal sister chromatid exchange, because a dicentric and an acentric chromosome would have resulted from such an event. In fact, each time the $V_\beta 14$ gene segment is used because of its inverted translational orientation, it should be joined by chromosomal inversion and there should be a flank recombination product present in that cell. It is interesting that in immunoglobulin genes, the reciprocal joints apparently do not accumulate at the λ or heavy-chain gene loci with the same frequency with which they are found at the κ locus^{18,25}. In fact, the feasibility of an inversion model for κ -gene joining has recently been established by the *in vitro* joining experiments of Lewis *et al.*^{18,25}. Because we have demonstrated that the presence of a V_β - D_β reciprocal joint is a consequence of the topological relationships between β -gene segments, we believe that in a similar way some V_κ gene segments will be found in an inverted orientation either 5' or 3' relative to the C_κ gene.

The T-cell receptor β -chain locus constitutes a striking example in which the gene segments to be joined have different transcriptional polarities. How many additional V_β gene segments have the same transcriptional polarity as the $V_\beta 14$ gene segment? To date, a number of T cells have been examined for all of their T-cell receptor gene rearrangements. However, analyses of 15 helper T-cell clones (refs 35, 37 and J. Kober and N.

Shastri, unpublished data) did not reveal any flank recombination products, a finding consistent only with V_β - D_β joining occurring via the looping out-excision or the sister chromatid exchange models. Therefore, we believe that most V_β gene segments will be in the same transcriptional polarity as the D_β - J_β - C_β gene clusters.

The β genes of the human T-cell receptor have been located on the long arm of chromosome 7, band q35 (refs 38, 39). Cytogenetic studies of lymphocyte cultures stimulated with T-cell mitogens and derived from patients with ataxia telangiectasia indicate increased chromosome breakage and various abnormalities of chromosomes 7 and 14. Particularly interesting is the observation⁴⁰ of a pericentric inversion of chromosome 7 with breakpoints at p13 and q35. It is tempting to speculate, in relation to our data, that this chromosome inversion mimics a normal site-specific V_β - D_β rearrangement by inverted joining. It should be noted with regard to this possibility that the t(11;14) chromosomal translocation recently observed in two separate cases of B-cell neoplasms has involved a heavy-chain segment

on chromosome 14 and a heptamer-nonamer signal-like sequence on the normal chromosome 11⁴⁰. In a similar way, a mouse nonproductive κ rearrangement has been shown to involve the joining of a J_κ segment to a sequence unrelated to any V_κ , which is flanked in the germ line by a recombination signal sequence resembling those found to the 3' side of V gene segments³⁰. Whether the pericentric inversion of chromosome 7 observed in ataxia telangiectasia involves the T-cell receptor β -chain locus and is mediated by a mechanism similar to the V_β - D_β inversion mechanism described in this article will require the molecular characterization of the chromosomal breakpoints.

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Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution

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The only well-understood mechanism of zymogen activation is that of the serine proteinases, in which proteolytic cleavage leads to conformational changes resulting in a functional active site. A different mechanism is now unveiled by the crystal structure of pepsinogen. Salt bridges that stabilize the positioning of the N-terminal proenzyme segment across the active site of pepsin are disrupted at low pH, releasing the amino-terminal segment and thereby exposing the catalytic apparatus and the substrate-binding sites.

THE specialized cells and tissues of eukaryotic organisms which produce proteolytic enzymes protect themselves from digestion by synthesizing inactive precursors of the active enzymes¹. These zymogens have lengths of polypeptide that are N-terminal extensions of the active proteinases. Activation of a zymogen is commonly accomplished by limited proteolysis that removes the proenzyme segment extension. Many biologically important processes, such as the blood clotting cascade, are critically dependent upon this precursor to active enzyme conversion².

The zymogens of the pancreatic serine proteinases are usually activated upon cleavage by other proteolytic enzymes. For example, the removal of an N-terminal hexapeptide from trypsinogen by enterokinase or trypsin is accompanied by a conformational change that completes the formation of the primary substrate-binding cavity of trypsin³. In contrast, the zymogens of the gastric aspartic proteinases only require H⁺ ions (pH <5.0) to initiate the activation process⁴. These pioneering observations of Herriott indicated that at pH 4.0-4.6 the formation of pepsin from the inactive precursor is autocatalytic.

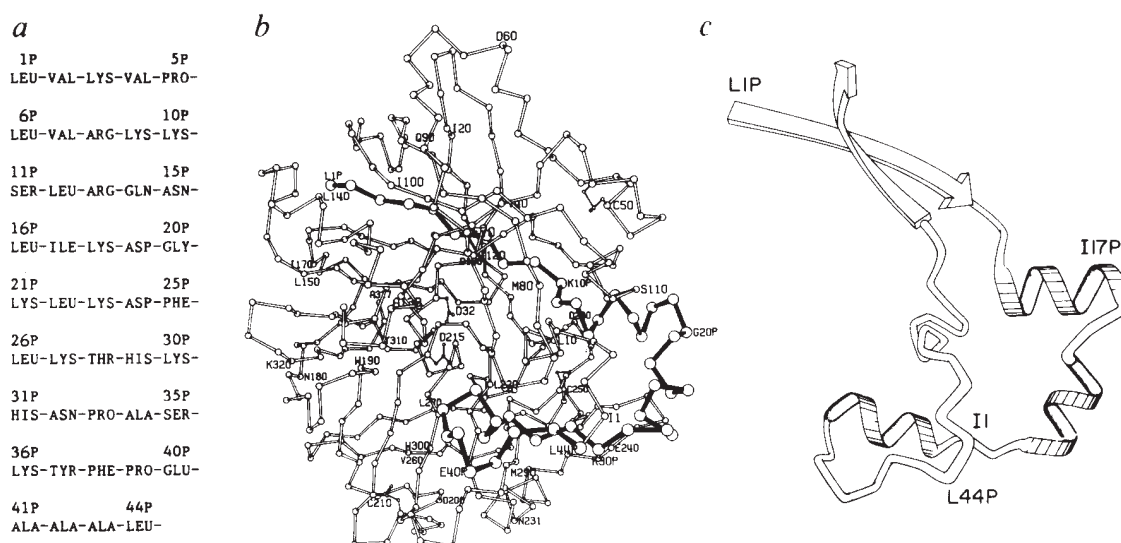


Fig. 1 *a*, Amino-acid sequence of the proenzyme segment of pepsinogen. The 44 residues are numbered sequentially from 1 followed by the suffix P as suggested by Foltmann¹³. The residues of the active portion of the molecule are numbered according to the porcine pepsin amino-acid sequence^{14,15}. *b*, Representation of the α -carbon atom positions of porcine pepsinogen (ORTEP, C. Johnson, Oak Ridge National Laboratories). The 44 residues of the proenzyme segment have the virtual bonds that connect C α atoms in thick black lines; the pepsin portion of the molecule is indicated by open bonds. The side-chain positions of Asp 32, Asp 215 and the three disulphide bridges are indicated. Every 10th residue is labelled with the single-letter code for the residue name and the sequence number. Large crystals of porcine pepsinogen suitable for high-resolution crystallographic analysis were grown by the hanging-drop, vapour-diffusion method. The protein solution contained 10 mg ml⁻¹ pepsinogen (Sigma, lot 98C-0451), 1.8 M Li₂SO₄ buffered with 50 mM [*N*-morpholino]ethane sulphonic acid to a pH of 6.1. The crystals of the native protein are monoclinic, space group C2, with unit cell dimensions $a = 105.8$, $b = 43.4$, $c = 88.6$ Å and $\beta = 91.4^\circ$. There are four molecules of pepsinogen, relative molecular mass $\approx 39,600$, per unit cell ($V_m = 2.57$ Å³ per dalton). The crystals used for this structure solution are similar to those of porcine pepsinogen reported previously²⁰ except for a 1% difference in the a axial length. Intensity data to 1.8 Å resolution (37,575 unique reflections) for the native pepsinogen crystals were collected on an Enraf-Nonius CAD4 diffractometer using a data collection protocol described previously²¹. The structure was solved by a combination of multiple isomorphous replacement²² and molecular replacement²³ techniques. The molecular replacement used a search model based on the 1.8 Å resolution refined coordinates of penicillopepsin¹⁹. Details of this structure solution will be reported elsewhere (A.R.S., M. Fujinaga, R. J. Read and M.N.G.J., in preparation). *c*, A ribbon drawing of the secondary structural features of the proenzyme segment (residues Leu 1P-Leu 44P of pepsinogen) along with the first 17 residues of active pepsin. Two of the scissile bonds cleaved during zymogen processing, Leu 16P-Ile 17P and Leu 44P-Ile 1, are labelled. There are three short helical stretches in the proenzyme segment, Ser 11P-Asp 19P, Lys 21P-Thr 28P, approximately orthogonal to one another, and Pro 33P-Tyr 37P. The side chains of Leu 12P, Leu 16P, Leu 22P, Phe 25P and Leu 26P contribute to the hydrophobic core of this small domain. The chain from His 29P to Asn 32P has an extended conformation. The remaining segment of the proenzyme peptide, from Pro 39P to Leu 44P, seems to have few secondary structural features. The electron density for the segment from Ala 42P to Ile 1 is relatively weak at the present refinement stage. The peptide bond between Leu 44P and Ile 1 is exposed on the surface of the molecule.

Many elegant chemical, spectrophotometric and kinetic studies have been designed to elucidate the pathway of the conversion of porcine pepsinogen to pepsin. Optical rotatory dispersion studies of pepsinogen⁵ showed that the zymogen exhibits characteristics of α -helical structure not present in pepsin. Furthermore, the conformation of the zymogen is stabilized by electrostatic interactions involving the basic amino acids of the proenzyme segment and carboxylate groups on the enzyme⁵. Ultraviolet spectroscopy detects a rapid conformational change in the molecule as a solution of pepsinogen is acidified to pH 2.85 (ref. 6). If the pH of the solution is returned to neutrality after a short time, this conformational change is fully reversible and no formation of pepsin is observed; this is consistent with a possible intermediate in the reaction⁶. It has also been shown that the activation of pepsinogen in a dilute acidic solution containing an excess of substrate, haemoglobin, is independent of pepsinogen concentration, suggesting an intramolecular activation process in which the unproteolysed zymogen cleaves itself⁷. Similar deductions of a unimolecular mechanism have been made independently by Funatsu *et al.*^{8,9}. Further support for an intramolecular activation comes from kinetic experiments showing that the rate of conversion of pepsinogen is a first-order reaction at pH values below 3, that is, independent of zymogen concentration¹⁰. At higher pH values, however, the observed kinetics are consistent with a mixed reaction mechanism in which pepsin-catalysed activation predominates¹⁰.

The proenzyme segment of pepsinogen consists of 44 amino acids of known sequence^{11,12} (Fig. 1*a*). It has not been possible to isolate the intact peptide Leu 1P-Leu 44P from activation solutions. Instead, several shorter peptides resulting from multiple proteolytic cleavages can be isolated, suggesting a sequential mechanism for the conversion. The first peptide to be observed results from the hydrolysis of the Leu 16P-Ile 17P bond, with the concomitant formation of an enzymatically active pseudopepsin molecule^{16,17}. The subsequent formation of mature pepsin is concentration dependent and may represent the bimolecular component important in the activation process at relatively higher pH values¹⁸.

Comparison of the crystallographic solution of the molecular structure of pepsinogen with that of an active aspartic proteinase, penicillopepsin¹⁹, reveals the reasons for the catalytic incompetence of the intact zymogen. In addition, the observed structure (Fig. 1*b, c*) sheds new light on the interpretations of the many solution studies.

Molecular structure of pepsinogen

The model of pepsinogen reported here has been subjected to restrained-parameter least-squares refinement²⁴, following the same strategy used in this laboratory for other protein structures²⁵. Whereas detailed hydrogen bonding interactions may change on further refinement and inclusion of all ordered solvent, interpretations based on the present structure should not change substantially. The high quality of the electron density

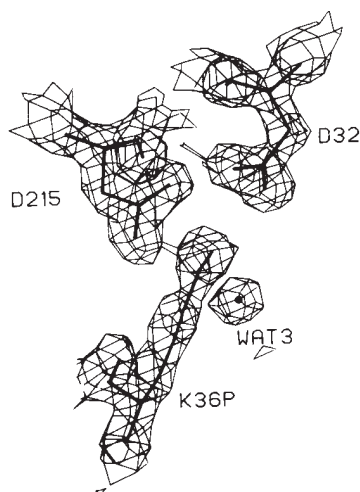


Fig. 2 Electron density contour surfaces in the vicinity of the active site of pepsinogen (Asp 32 and Asp 215). The current agreement factor between observed structure factor amplitudes, $|F_o|$, and calculated ones based on the refined atomic coordinates, $|F_c|$, is 0.167 ($R = \sum ||F_o| - |F_c|| / \sum |F_o|$) for the 26,191 reflections with $I \geq 2\sigma(I)$ in the 6.0–1.8-Å resolution range (I is the measured intensity of the reflection, $\sigma(I)$ its estimated standard deviation). The molecular model includes 110 ordered solvent molecules. The root-mean-square deviations from expected geometry are acceptably small, 0.016 Å for covalent bond lengths, 0.037 Å for bond angle-related distances, 0.025 Å for out-of-plane deviations and 4.0° for deviations from 180° for the *trans* peptide bonds. The contour level on this $2F_o - F_c$ map is 0.60 eÅ⁻³. Full thick lines correspond to the molecular model at this stage of the refinement. Solvent molecule Wat 3 is hydrogen bonded to N^ε of Lys 36P.

map at the current stage of refinement can be seen in Fig. 2. The present agreement factor is 0.167.

The crystallographic structures of four aspartic proteinases have been reported previously^{19,26–28}. All four structures are highly homologous and have a general overall shape that resembles a croissant. The N-terminal domains, residues 1–170, are folded in a similar fashion to the C-terminal domains (residues ~180–327), suggesting gene duplication, divergence and subsequent fusion in the evolutionary history of these enzymes²⁹. Most residues of the aspartic proteinases adopt a β -sheet conformation. The secondary and tertiary structural interactions in penicillopepsin at 1.8 Å resolution have been discussed elsewhere¹⁹. In all of these enzymes, the active-site aspartate residues, Asp 32 and Asp 215, are located at the junc-

tion of the two domains in an extended substrate-binding cleft. Possible modes of productive binding of substrates have been established by crystallographic studies of inhibitor binding to penicillopepsin^{21,30–32} and to rhizopuspepsin^{33,34}.

The polypeptide chain of the pepsin portion of pepsinogen (residues Ile 1–Ala 327) folds in a fashion that is highly homologous to those of the several aspartic proteinases of known structure mentioned above. Visual comparison of the α -carbon atom chain tracing of pepsinogen (Fig. 1b) with that of the active pepsin molecule²⁸ certainly confirms this. A more detailed comparison must await the completion of the refinement of both molecular structures. Nevertheless, there is clearly a major conformational difference between the N-terminal 12 residues of pepsin and the corresponding 12 residues in pepsinogen. In pepsin, residues Ile 1–Leu 6 form strand 1 of a large six-stranded antiparallel β -sheet, a common feature of all aspartic proteinases¹⁹. The next five residues form a reverse turn that changes the polypeptide chain direction²⁸.

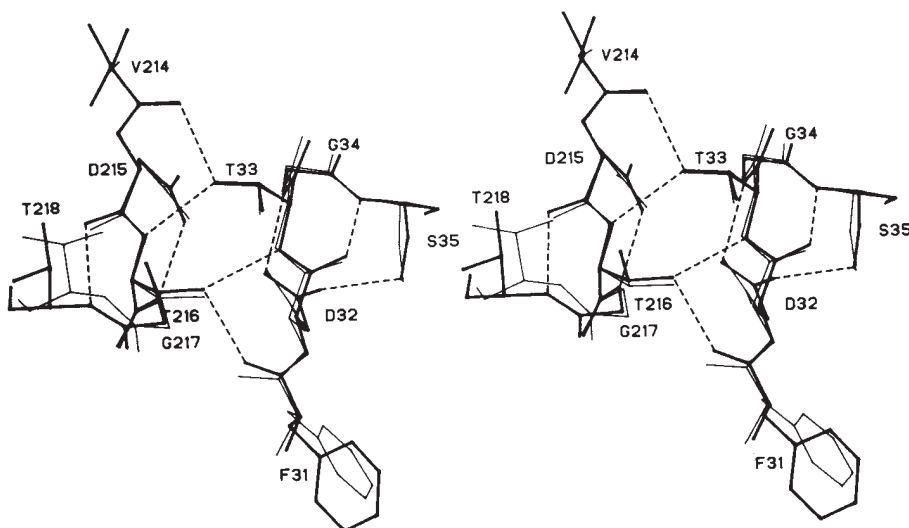
In contrast, these residues in pepsinogen are intimately associated with the inactivation domain, on the opposite side of the molecule (Fig. 1b). The side chain of Ile 1 in the zymogen is ~40 Å from its final position in the active enzyme. Residues Ile 1–Asp 3 are in an extended conformation, followed by a series of reverse turns from Glu 4 to Asp 11 which give the chain an irregular helical conformation. From Pro 39P to Asn 8, the polypeptide chain forms a large loop into the centre of which fits the tip of the flap (a β -hairpin loop, residues 72–82). The side chain of Leu 10 contributes to the hydrophobic core of the inactivation domain.

The overall shape of the inactivation domain of pepsinogen (residues Lys 9P–Glu 13) is that of a flattened disk with a long N-terminal extension formed by the first 8 residues, Leu 1P–Arg 8P (Fig. 1c). This disk-like domain fits neatly into the part of the structure that corresponds to the substrate-binding cleft in pepsin with apparently little distortion of the conformation of the active enzyme. The first six residues of pepsinogen form the first strand of the same large, centrally located six-stranded antiparallel β -sheet as do the N-termini of the active aspartic proteinases.

Mechanism of inhibition

The present molecular structure of pepsinogen clearly shows why the zymogens of the aspartyl proteinases are inactive. Part of the proenzyme segment, residues Ser 11P–Leu 44P, occupies the substrate-binding cleft of the pepsin portion of the zymogen, competitively blocking access to the two catalytically important aspartyl residues (Figs 1b, 2). Lys 36P has a pivotal role in the inhibition of the intact zymogen at high pH. This residue is in

Fig. 3 A stereo representation of the two active-site strands: Phe 31–Ser 35 and Val 214–Thr 218 of pepsinogen (drawn in thick lines), superimposed on the equivalent residues of penicillopepsin¹⁹: Phe 32–Ser 36 and Ala 212–Thr 216 (in thin lines). Hydrogen-bonding interactions involving atoms of pepsinogen are indicated by dashed lines. The least-squares superposition of the coordinates of the main-chain atoms (N, C α , C β , C, O) of these residues yields a root-mean-square deviation of 0.36 Å for the 48 common atom pairs. The largest difference is associated with the main chain of Thr 218 (Thr 216 in penicillopepsin). The hydrogen bond that the O^{γ1} side-chain atom of this residue forms with the side-chain carboxyl group of Asp 213 in penicillopepsin has no equivalent in pepsinogen. All other hydrogen-bonding interactions for this region of the zymogen are as observed for active penicillopepsin.



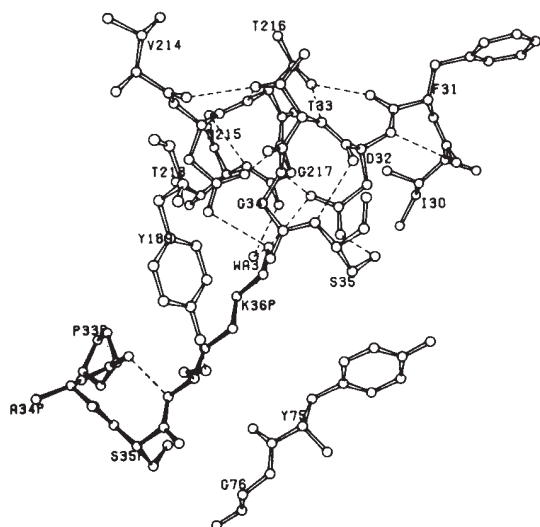


Fig. 4 A rendering of the active-site region of pepsinogen with four residues of the proenzyme segment, Pro 33P-Lys 36P (PLUTO, S. Motherwell, University Chemical Laboratory, Cambridge, UK). Hydrogen-bonding interactions are shown by dashed lines. The residues of the proenzyme segment are drawn with filled bonds, the pepsin portion with open bonds, both in this figure and in Fig. 5. The molecule labelled WA3 corresponds to water 3, depicted in Fig. 2 with its associated electron density.

a highly conserved region of the zymogens of the vertebrate aspartic proteinases³⁵.

We suggest that the residues of pepsinogen in the immediate vicinity of the two catalytic aspartyl residues are in, or very close to, their active conformation. It is not possible to consolidate this hypothesis by direct comparison with the known structure of pepsin²⁸. Crystals of porcine pepsin have been grown from ethanol, a compound known to inhibit this enzyme. Ethanol molecules have been tentatively identified in close proximity to the aspartyl residues, a binding that could induce conformational changes²⁸. In any event, only the pepsin α -carbon coordinates are available in the Brookhaven Protein Data Bank³⁶. Another member of the aspartyl proteinase family, penicillopepsin, has had its crystal structure determined at its pH optimum. The details of the conformation of the active site, with its intricate hydrogen bonding, have been described elsewhere¹⁹. A least-squares superposition of the active-site regions of penicillopepsin and pepsinogen is depicted in Fig. 3. The small rotation of the side chain of Asp 32 relative to the native conformation of penicillopepsin, as can be seen in Fig. 3 for pepsinogen, has also been observed in the crystal structure of the complex of penicillopepsin with the inhibitor Iva-Val-Val-StaOEt (refs 21, 30).

In the active conformation of penicillopepsin, the two carboxyl groups of Asp 33 and Asp 213 are the recipients of several strong hydrogen bonding interactions¹⁹. Asp 32 and Asp 215 in pepsinogen have most of these hydrogen bonds present (Fig. 3) but, in addition, the positively charged N^{ϵ} of Lys 36P provides electrostatic stabilization through hydrogen bonding for one of the formal net negative charges on these residues (Fig. 4). This interaction must also be an important factor in the inhibition of the inherent activity of the zymogen. It is likely that the net charge at the active site of pepsinogen is -2 , as the pH of the crystals is 6.1 and the higher pK_a of the pepsin active site is 4.7. Stabilization of the second formal negative charge must come from the other favourable hydrogen bonds and dipole interactions. The position of N^{ϵ} of Lys 36P relative to the two aspartic acid side chains in pepsinogen is within 0.5 Å of the refined position of a solvent molecule, 039, in penicillopepsin. We have

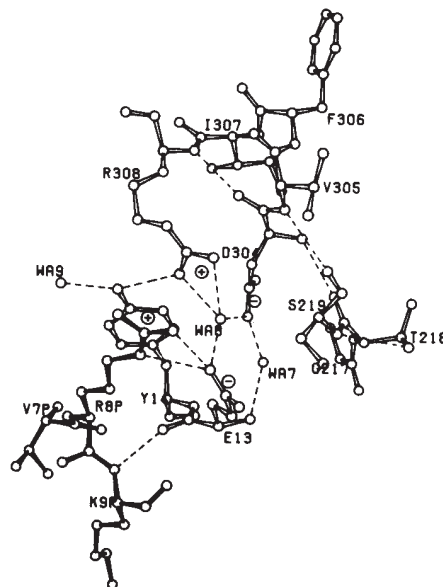


Fig. 5 The residues neighbouring the unusual interaction of Arg 8P and Arg 308 are shown (PLUTO, S. Motherwell, University Chemical Lab., Cambridge, UK). Possible hydrogen bonds are represented by dashed lines. The close proximity of the carboxylate groups of Glu 13 and Asp 304 are important in stabilizing the disposition of the two arginine residues at high pH. Protonation of Glu 13 and Asp 304 on lowering the pH below 4 would remove these stabilizing electrostatic effects and strong repulsion of the two positively charged guanidinium groups could trigger a conformational change. Water WA8 bridges the interaction between Asp 304 and Arg 308.

interpreted this solvent as a bound ammonium or hydronium ion with a possible electrophilic role in the catalytic mechanism³². The disposition of Lys 36P provides additional evidence that the 039 site of penicillopepsin is a favourable position for the binding of positively charged groups to aspartyl proteinases, an important yet unresolved issue in the elucidation of the mechanism of the active enzymes^{19,32}.

There are four aromatic rings in the vicinity of this electrostatic interaction of Lys 36P and the two aspartic acid carboxyl groups, Tyr 37P, Tyr 9, Tyr 75 and Tyr 189. The side chain of Tyr 75 on the flap is stacked with its aromatic plane almost perpendicular to that of Tyr 9. One of the first events detected spectrophotometrically on lowering the pH of a solution of pepsinogen from neutrality to 2–4 is an ultraviolet spectral change that can be associated with tyrosyl and tryptophan absorbance bands⁶. As the active-site carboxyl groups take up a proton, the complicated hydrogen bonding network involving Asp 32 and Asp 215 would be disrupted with a concomitant alteration in the environment of these several tyrosine side chains, as observed in the spectrophotometric experiments.

Intramolecular ionic interactions

It has been suggested that, at neutral pH, the highly positively charged proenzyme segment is bound to the pepsin portion of the molecule via ionic interactions with negatively charged carboxylate groups⁵. This early suggestion is now confirmed by the crystal structure. In addition to the ionic attractions between the Lys 36P side chain and the carboxylate groups of Asp 32 and Asp 215 that we have discussed (Fig. 4), several other electrostatic interactions contribute to this stabilization (Table 1).

A cluster of two positively charged groups (Arg 8P and Arg 308) and two negatively charged carboxylate groups (Glu 13 and Asp 304) is shown in Fig. 5. Also included in the environment of these charged groups are several highly ordered water

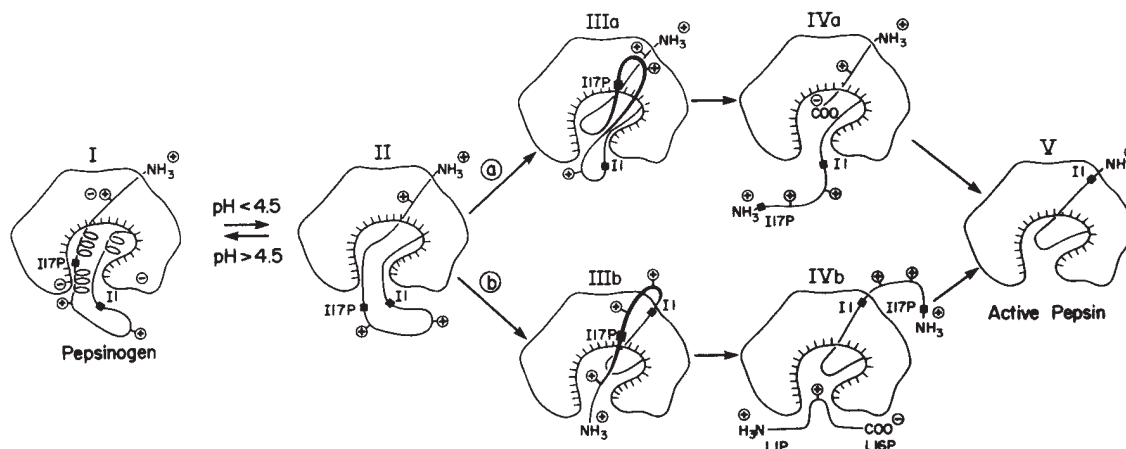


Fig. 6 Proposed steps in the activation of pepsinogen. Interconversion of structures I and II is a reversible, pH -dependent process. Structures IIIa and IIIb are two possible alternatives that would place the first scissile bond Leu 16P-Ile 17P at the catalytic site. In pathway *a*, the N-terminus of the zymogen remains in its crystallographically observed position; in pathway *b* the N-terminal residues of pepsin (Ile 1-Asn 8) occupy their final position by replacing the N-terminus of the proenzyme segment. The segment of polypeptide chain from Ile 17P to Ile 1 is sufficiently long to wrap around the back of the molecule, allowing the scissile bond Leu 16P-Ile 17P to approach and bind in a productive mode analogous to that shown in structure IIIa. A unimolecular reaction would release peptide Leu 1P-Leu 16P to yield structures IVa or IVb. This peptide is a known inhibitor of porcine pepsin³⁷ and therefore probably also inhibits pseudo-pepsin, as indicated schematically in structure IVb. Conversion of structure IVa or IVb to active pepsin probably occurs by an intermolecular pepsin cleavage to release peptide Ile 17P-Leu 44P.

molecules, WAT 7, 8 and 9, which mediate additional interactions. The positively charged guanidinium groups of the two arginine side chains stack with their planes approximately parallel, but separated by a van der Waals contact distance of ≈ 3.5 Å. This surprising interaction is stabilized by the close proximity of the two carboxylate groups of Glu 13 and Asp 304 (Fig. 5). A possible sequence of events on lowering the pH of a solution of pepsinogen is the protonation of these carboxylate groups, thus removing the charge stabilization. The two guanidinium side chains would be electrostatically repelled, inducing a changed conformation in the proenzyme segment. Similar arguments apply to protonation of the several other carboxylate groups listed in Table 1. This leads to the disruption of the conformation of the zymogen as the pH is lowered.

Steps in activation

The molecular structure of the zymogen of porcine pepsin places limitations on the sequence of events required to convert the inactive precursor to the activated enzyme. Structure I in Fig. 6 is a diagrammatic representation of the conformation of pepsinogen that we have determined crystallographically at $pH > 6$. On lowering the pH of a solution of pepsinogen, conversion of structure I to structure II (Fig. 6) is accompanied by the following known events. The carboxylate groups involved in stabilizing ion pairs (Table 1) become protonated, facilitating a conformational change in the proenzyme segment. This change can be

fully reversed by raising the pH of the solution after a short time⁶. However, prolonged incubation results in irreversible activation with the intramolecular cleavage of the peptide bond Leu 16P-Ile 17P^{16,17}, a bond favoured for hydrolysis because pepsin has a specificity for hydrophobic side chains³⁸. This scissile bond is part of the first helix of the proenzyme segment (Fig. 1) and residues Leu 16P and Ile 17P must move ≈ 18 Å to reach the active site. In addition, this bond must approach the two aspartate groups Asp 32 and Asp 215 to bind in the manner of a good substrate^{31,32}.

There are two ways in which the scissile bond Leu 16P-Ile 17P can be positioned in the substrate-binding cleft (pathways *a* and *b* of Fig. 6). Structure IIIa results when Leu 1P-Leu 6P, the extended polypeptide preceding the first helix of the proenzyme segment, remains in the zymogen conformation as part of the six-stranded antiparallel β -sheet (Fig. 1). The N-terminal to C-terminal direction of the polypeptide corresponds to that proposed for productive substrate binding to aspartyl proteinases^{31-34,39}. The second mode of binding, represented by structure IIIb, occurs if the segment Leu 1P-Leu 6P is displaced by the N-terminal residues of pepsin (Ile 1-Leu 6). In both cases, intramolecularly catalysed cleavage of the bond Leu 16P-Ile 17P would yield structures IVa and IVb, respectively.

The most likely path in the conversion of intermediate structures IVa and IVb to active pepsin, structure V, is via an intermolecular cleavage step in which the peptide bond Leu 44P-Ile 1 is bound in the active site of a second activated pepsinogen (or active pepsin) molecule. These two structures, IVa and IVb, are quite analogous and are differentiated only by how peptide Leu 1P-Leu 16P is bound to the enzyme. Further degradation of the two major peptides released during the activation process takes place, resulting in many short segments³⁷.

The activation of mammalian aspartic proteinases from their inactive precursors is accompanied by some spectacular conformational changes. We have shown that the residues in the active site of pepsinogen are in a conformation remarkably close to the active conformation of penicillopepsin (Fig. 3). The pH -induced conformational upheavals involve the movement of the competitive proenzyme segment out of the active-site cleft of pepsin, exposing the catalytic apparatus and freeing the substrate-binding sites. The position of the N-terminal Ile 1 residue

Table 1 Electrostatic interactions of the proenzyme segment with the pepsin part of pepsinogen

Positively charged residue*	Negatively charged residue	Hydrogen-bonding distances (Å)
Lys 3P ϵ -NH ₃ ⁺	Asp 171 COO ⁻	3.5
Arg 8P Guan ⁺	Glu 13 COO ⁻	2.9, 2.8
Arg 13P Guan ⁺	Asp 11 COO ⁻	2.8, 2.8
His 29P Imid ⁺	Asp 3 COO ⁻	2.8
His 31P Imid ⁺	Glu 7 COO ⁻	2.5
Lys 36P ϵ -NH ₃ ⁺	Asp 32 COO ⁻	3.0
	Asp 215 COO ⁻	2.8

* Guan⁺ and Imid⁺ stand for the guanidinium and imidazolium groups of arginine and histidine, respectively.

of active pepsin is $\approx 40 \text{ \AA}$ from the observed position of this residue in pepsinogen. The activation steps we propose in order to rationalize how this remarkable conformational change can take place are entirely consistent with the low pH unimolecular activation process that has been suggested to occur in solution.

Dr Y. Nakagawa supplied us with our initial sample of highly

purified porcine pepsinogen. Koto Hayakawa has grown the pepsinogen crystals and carried out the heavy-atom search. Randy Read and Masao Fujinaga provided their expertise with the molecular replacement and phase combination methods. This research has been generously supported by the MRC of Canada in a grant to the MRC Group in Protein Structure and Function.

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LETTERS TO NATURE

A radio outburst and jet from the symbiotic star CH Cyg

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CH Cyg is an M7 III star that is known to undergo periods of brightening in the blue-violet continuum at irregular intervals of a few years¹⁻³. During the active phase, the spectrum also exhibits strong Balmer emission and emission lines from He I, Fe II and various forbidden transitions, giving it the appearance of a symbiotic star. As part of a monitoring programme on symbiotic stars, observations with the National Radio Astronomy Observatory Very Large Array (VLA) have been made on CH Cyg over the past year at intervals of a few months. During the period April 1984 to May 1985 this monitoring revealed a strong radio outburst coincident with the production of a multi-component jet, and a comparison of high-resolution maps at two epochs separated by 75 days shows that the jet is expanding at a rate of $1.1 \text{ arc s yr}^{-1}$. The onset of the radio outburst coincides in time with a remarkable decline in the visual brightness of the star. The luminosity of the star during the optically active state and the energetics of the mass loss suggest that the outburst is the result of accretion at, or near, the Eddington limit onto the surface of a white dwarf.

The observations of CH Cyg trace the evolution of a radio outburst during which the flux density increased by a factor of 30. Figure 1 shows the radio light curve at $\lambda = 2 \text{ cm}$. The outburst was continuing at the time of our last observation on 3 May 1985, and the radio spectrum during the outburst has remained essentially constant. Figure 2 shows radio spectra obtained at three epochs. The spectral index between 5 and 14.9 GHz is

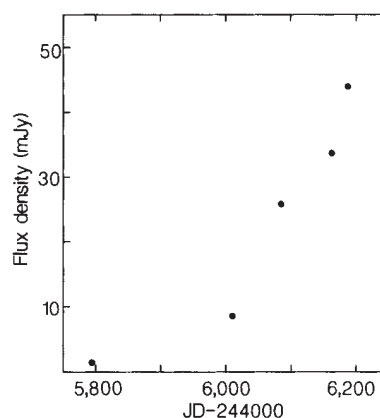


Fig. 1 The radio light curve of CH Cyg at $\lambda = 2 \text{ cm}$ from April 1984 to May 1985.

+1.2. This is typical (although slightly high) for radio-detected symbiotic stars⁴, and is consistent with emission through optically thick thermal bremsstrahlung.

The radio brightening illustrated by Fig. 1 is reminiscent of symbiotic novae. About six such objects are known, for example, HM Sge, V1016Cyg, RR Tel, and high-resolution maps show shell-like geometries on the sub-arc second scale^{5,6}. In contrast, our high-resolution maps of CH Cyg reveal a jet-like feature. Moreover, the 'jet' is clearly expanding at a high velocity. This effect is shown in Fig. 3, where we present maps of CH Cyg at $\lambda = 2 \text{ cm}$ obtained at two epochs with the highest resolution configuration of the VLA. The Smithsonian Astrophysical Observatory catalogue position of CH Cyg, with 1σ error bars, is shown by the cross on each map. The first map from 8 November 1984 shows two radio components (labelled A and C) with roughly equal flux density separated by 0.18 arc s . On 22 January 1985, 75 days later, the source exhibits an additional component (B), and the separation of A and C appears to have increased to 0.4 arc s . The position of the midpoint between A

and C on the two dates agrees to within 0.02 arc s, which is the same to within the accuracy of the measurements and implies that the jet feature is a bipolar phenomenon originating from a point near the position of component B. Extrapolating the rate of expansion backwards in time indicates that components A and C were produced between 22 July and 7 September 1984. This agrees well with the time of onset of radio outburst shown in Fig. 1, and is about 5 months after our first observation in April 1984. This earlier detection might thus be interpreted as a quiescent level of emission or an earlier, weak outburst.

The position, flux density and angular extent of each component in Fig. 2, based on elliptical gaussian fits, are listed in Table 1. From the flux densities and angular sizes, the brightness temperature at 14.9 GHz is about the same for each component

Table 1 CH Cyg jet components

	RA(1950)	Dec.(1950)	S(14.9 GHz) (mJy)	a_{maj} (arc ms)	a_{min} (arc ms)	PA (°)
8 November 1984						
A	19 23 14.121	50 08 30.79	4.2 ± 0.3	71 ± 7	55 ± 8	115 ± 10
C	14.133	30.65	5.1 ± 0.3	75 ± 5	30 ± 9	175 ± 15
22 January 1985						
A	14.111	30.88	6.2 ± 0.16	94 ± 2	74 ± 3	95 ± 7
B	14.123	30.76	12.2 ± 0.18	196 ± 2	66 ± 4	141 ± 1
C	14.139	30.59	7.4 ± 0.17	146 ± 2	52 ± 4	149 ± 1

and equal to 7×10^3 K, consistent with the equilibrium temperature of a photoionized gas cooled primarily by forbidden line emission. The optical depth of a fully ionized gas at radio wavelengths is given by

$$\tau(\nu) = 2.7 \times 10^{-20} \nu^{-2.1} T_e^{-1.35} \int_0^w n_e^2 dl$$

where ν is the radiation frequency (GHz), T_e and n_e are the electron temperature (K) and density (cm^{-3}), w is, in this case, the width of the jet (cm) and l is the path link. Estimates of the distance to CH Cyg range from less than 200 to 600 pc. Adopting an intermediate value of 400 pc, the typical component width of 0.06 arc s on 22 January, corresponds to $w = 3 \times 10^{15}$ cm. Because the jet is partially optically thick up to 22 GHz, under the assumption of cylindrical symmetry, we can place a lower limit of $2 \times 10^6 \text{ cm}^{-3}$ on the r.m.s. electron density, yielding masses $M_A > 6 \times 10^{-7} M_\odot$, $M_B > 1.3 \times 10^{-6} M_\odot$ and $M_C > 5 \times 10^{-7} M_\odot$. For distance $D = 400$ pc the rate of elongation of the jet corresponds to a projected velocity of $2,100 \text{ km s}^{-1}$. The bipolar expansion velocity is thus $V_e = 1,050/\sin i \text{ km s}^{-1}$, where i is the inclination of the jet axis to the line-of-sight.

Figure 4 shows the visual light curve of CH Cyg observed by the American Association of Variable Star Observers (AAVSO) from April 1981 to May 1985. Beginning about 1977 the star had been in an optically active state, reaching a peak visual magnitude of about 5.8. This is the highest brightness level observed since monitoring began in 1935. However, the start of the radio outburst coincides with a remarkable decline of 1.5 mag in the visual brightness to the pre-1977 level. This decline of July 1984 was also noted by Tomov⁷, who attributed it to a decrease in the blue continuum source based on the fact that the TiO molecular bands of the red giant, which are almost completely filled in by the continuum in earlier spectra, are clearly visible in August and September. He also notes that the Balmer emission had become very wide, expanding to $\sim 2,500 \text{ km s}^{-1}$ in H β . Selvelli and Hack⁸ also observed a large increase in the Balmer line widths between July and November 1984. They report a full width of $3,950 \text{ km s}^{-1}$ for Ly α in November 1984 and January 1985.

We have considered two possible explanations for the expanding jet feature: (1) the propagation of an ionization front from a highly directional source of ionizing photons; and (2) the outward motion of ionized material along an axis defined by the central power source. The sudden turn on of a hot source that emits L_{ph} ionizing photons per second will produce an

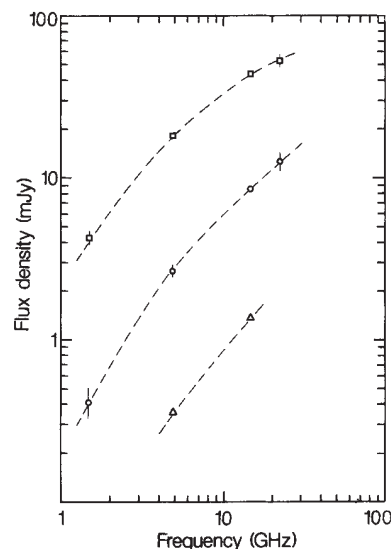


Fig. 2 Radio continuum spectra of CH Cyg at three epochs: $\square$, 3 May 1985; $\circ$, 8 November 1984; $\triangle$, 6 April 1984.

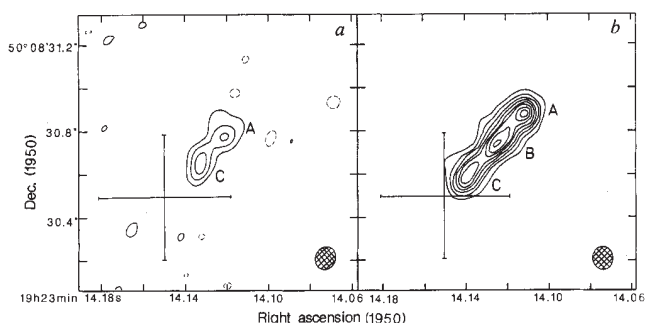


Fig. 3 VLA radio maps at $\lambda = 2$ cm separated by 75 days (a, 8 November 1984; b, 22 January 1985) showing the evolution of the jet. The half-power ellipse of the synthesized beam is indicated at lower right.

ionization front propagating outward at a velocity roughly given by $v = L_{\text{ph}}/4\pi r^2 n_H$, where n_H is the constant number density of hydrogen atoms. Taking $n_H = 2 \times 10^6 \text{ cm}^{-3}$ and L_{ph} sufficient to ionize material to an angular distance of 0.2 arc s yields $v \approx 2,000 \text{ km s}^{-1}$ for $D = 400$ pc. A model of this type can thus account for the observed expansion rate. It seems, however, unlikely that a rapidly propagating front should be produced at a time when the optical luminosity is decreasing; although it is possible that most of the flux has shifted to the ultraviolet, as is observed during the optical decay of novae. Another difficulty is that, in the case of a propagating front, one expects the structure of material behind the ionization front to remain roughly constant. Such a model is therefore difficult to reconcile, in a straightforward manner, with the appearance, at a later date, of a third component interior to the front. If the material of component B were present on 8 November, it should have been visible.

The radio data thus favour a material flow model. Such an interpretation is further strongly supported by the appearance of high-velocity Balmer emission at the time of the onset of the optical decline and radio outburst. If the radio jet represents outflowing material, a lower limit on the rate of mass ejection is provided by the fact that component B is absent on 8 November. The mass M_B must have been ejected over an interval of < 75 days, yielding a mass ejection rate $\dot{M}_e > 7 \times 10^{-6} M_\odot \text{ yr}^{-1}$ for $V_e > 1,050 \text{ km s}^{-1}$. The energy flow involved in the mass loss

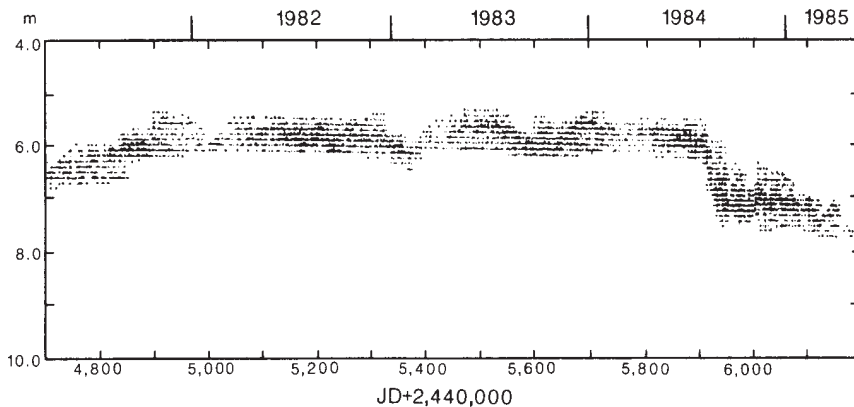


Fig. 4 The AAVSO visual light curve of CH Cyg from April 1981 to May 1985. Each dot represents one observation.

is $>3 \times 10^{36} \text{ erg s}^{-1}$, and the total kinetic energy of the bulk motion is $\geq 10^{43} \text{ erg}$. The energetics of the mass flow is thus similar to that observed in nova shells, and points to an origin in an event having total luminosity of the order of at least $10^{37} \text{ erg s}^{-1}$. Luud *et al.*⁹ pointed out that the integrated ultraviolet flux measured in September 1981 yields a luminosity of $\sim 10^{38} \text{ erg s}^{-1}$. The total radiative luminosity at maximum light is thus sufficient to power the outflow.

It has been suggested previously that the CH Cyg system contains an accretion disk^{10,11}. Most notably, Slovak and Africano¹⁰ have observed optical continuum flickering on a timescale of a few minutes. Such flickering is common among cataclysmic variables, where it is attributed to a hotspot on an accretion disk around a white dwarf. In the case of CH Cyg, the timescale of the flickering implies an upper limit of $\sim 10^{10} \text{ cm}$ in the radius of the accreting object. We are therefore led to the suggestion that the energy required for the creation of the jet feature is produced by accretion onto the surface of a compact object. For a $1 M_{\odot}$ object with radius R the critical rate at which the accretion-generated luminosity exceeds the Eddington limit is given by

$$\dot{M}_{\text{cr}} \approx 10^{-3} \left(\frac{R}{R_{\odot}} \right) M_{\odot} \text{ yr}^{-1}$$

For $R < 10^{10} \text{ cm}$, $\dot{M}_{\text{cr}} \leq 10^{-4} M_{\odot} \text{ yr}^{-1}$, and for a white dwarf $\dot{M}_{\text{cr}} \approx 10^{-5} M_{\odot} \text{ yr}^{-1}$. These accretion rates are comparable to the mass loss rate in the jet. Since during an accretion driven outburst $\dot{M}_{\text{e}}$ will be less than or, at most, equal to the rate of accretion, $\dot{M}_{\text{acc}}$, onto the central object, we infer that for the event leading to the CH Cyg jet $\dot{M}_{\text{acc}} \geq \dot{M}_{\text{cr}}$. From the calculations of Shakura and Sunyaev¹², accretion through a disk at a rate close to, or exceeding, the Eddington limit can produce a radiation-driven outflow of matter along the angular momentum axis of the disk. Bath¹³ has modelled such an outflow and finds that outflow velocities of a few thousand km s^{-1} can be obtained from supercritical accretion onto a white dwarf, and expected mass ejection rates are $< 0.5 \dot{M}_{\text{cr}}$, in excellent agreement with the observations. The ultraviolet luminosity before the radio outburst suggests that the accretion rate has been near, or at, supercritical since 1981.

The formation of a massive disk and accretion rates of the order $10^{-5} M_{\odot} \text{ yr}^{-1}$ imply that mass transfer occurs through Roche lobe overflow from the red giant. Accretion rates from the red giant wind alone will be much too small, typically $10^{-8} M_{\odot} \text{ yr}^{-1}$ or less. A number of periods, both spectroscopic and photometric, have been reported for CH Cyg, ranging from about a month to many years. The fact that since 1977 CH Cyg seems to spend most of the time in an optically active state, that the luminosity has been near supercritical since at least 1981 and that flickering has been observed at many epochs, indicates that a high accretion rate persists for a large fraction of the orbital period. For a radius of the M7III primary of $R_{*} = 200 R_{\odot}$

(ref. 14), a maximum orbital separation of about $2R_{*}$ (for high mass transfer rate through the inner lagrangian point) would require an orbital period of $\leq 3 \text{ yr}$.

An object with radius typical of a white dwarf and luminosity $L \sim 10^{38} \text{ erg s}^{-1}$ will have a surface temperature $T = 10^5\text{--}10^6 \text{ K}$, and might be detectable as an X-ray source. In this regard, it is noteworthy that CH Cyg lies near the centre of the error box of the HEAO A-2 hard X-ray source H1926+503 (ref. 15). If the X-ray flux is associated with CH Cyg, the luminosity, measured in November 1977 when the source was entering the optically active state, is $\sim 10^{33} \text{ erg s}^{-1}$, consistent with optically thick accretion through a disk onto a white dwarf at a rate $\geq 10^{-7} M_{\odot} \text{ yr}^{-1}$ (ref. 16). X-ray observations of CH Cyg during the outburst will be very important.

The radio outburst from CH Cyg is an unusual and complex event, and, while we have presented a preliminary picture to account for the phenomenon, some problems remain. Although the observational evidence strongly supports a material flow model, a propagating ionization front cannot be ruled out. In either case, it is difficult to reconcile the optical decline with either the radiation required for the ionization or the energy for the mass flow. Further observations are clearly needed. A measurement of the ultraviolet luminosity will be particularly valuable. The spectral evolution during the decay of the radio outbursts will also provide important clues. The radio decay should be accompanied by a flattening of the radio spectral index as the material expands and becomes optically thin.

The only other symbiotic star known to exhibit a jet-like feature is R Aqr¹⁷. This feature, which appeared some time between 1970 and 1977, was first detected optically as an emission spike^{18,19}. An ejection model for this source²⁰ has been questioned because no proper motion of the components has been observed²¹. Spergel *et al.*²² have suggested instead that the appearance of the 'R Aqr jet' was the result of sudden photoionization of condensations in the red giant wind. More recently, Solf and Ulrich²³ have successfully modelled the kinematics of the nebula about R Aqr in terms of two episodes of bipolar shell ejection 185 and 640 yr ago, with total shell mass of $\sim 10^{-5} M_{\odot}$ and expansion velocity of the order of 100 km s^{-1} . The CH Cyg outburst provides the first opportunity to observe an event giving rise to a radio jet from a symbiotic star. It will be important to follow the evolution of the CH Cyg jet at radio and other wavelengths, both to clarify the nature of the outburst and jet formation and to study the kinematics of the jet components at large radii.

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Mechanism of stable jet formation in electrohydrodynamic atomization

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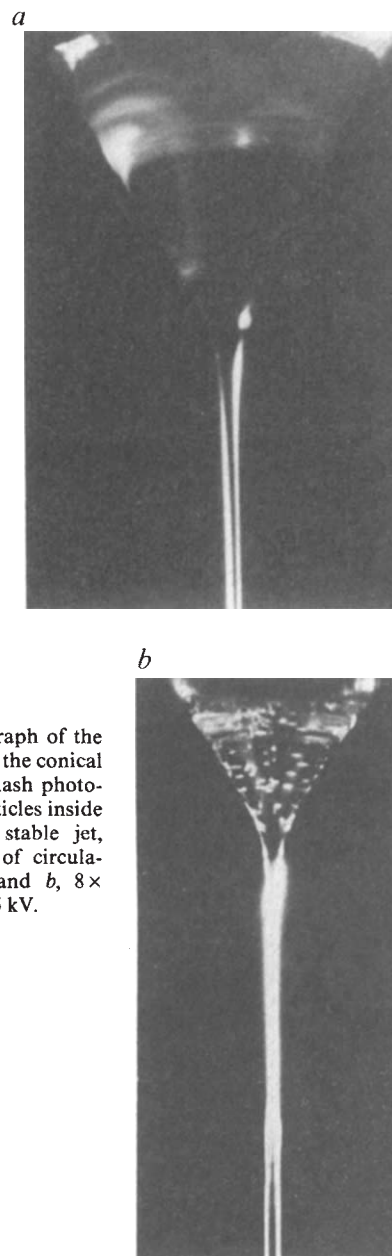
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The disruption of a liquid into a spray of charged droplets, when subjected to an intense electric field, has attracted considerable interest from both a fundamental and applied point of view. On the applied side, the process has been used in paint spraying, electrostatic printing, electrostatic emulsification, fuel atomization in combustion systems and in space vehicle propulsion systems. More recently, the phenomenon has found potential application in crop spraying because of the low energies required, the ability to produce fine sprays within a narrow size distribution and the preferential deposition on the target surfaces. Despite its wide range of applications, the mechanism of liquid disruption is only poorly understood. At a critical potential, the so-called Taylor¹ cone develops, with a fine stable jet issuing from its tip. The formation of such cones and jets is essential for the production of an electrohydrodynamic spray. Most previous work aimed at understanding jet formation has been based on high-speed photographic techniques. We have made direct observations of jet formation and our results, presented here, demonstrate the role of electrical shear effects in this process, and invalidate those theories that assume a uniform velocity profile² for the liquid in the conical base of the jet.

If a large potential (up to ~15 kV) is gradually applied to a capillary through which a semiconducting liquid is flowing slowly, the size of the emergent drop decreases while the dripping rate increases. At a certain potential the drop at the end of the capillary ejects a small drop or a long filament. This filament breaks off and the meniscus of the drop relaxes back to its initial shape in a pulsating fashion, the rate of which increases with an increase of potential. At a critical potential, depending on the conductivity of the liquid, the main drop develops into a cone—the Taylor¹ cone—with a fine stable jet

Fig. 1 a, Streak photograph of the circulation pattern within the conical base of a stable jet. b, Flash photograph of lycopodium particles inside the conical base of a stable jet, indicating the direction of circulation. Flow rate for a and b, $8 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ at 10.5 kV.



issuing from its tip; this is a prerequisite for the production of an electrohydrodynamic spray, which consists of a symmetrical assembly of such jets around the capillary or nozzle. Despite the importance of stable jets, their mechanism of formation remains unclear. It is known, for example, that not all liquids form such stable jets and that effective atomization can only be achieved if the conductivity of the liquid is within the limits 10^{-6} – $10^{-8} \Omega^{-1} \text{ m}^{-1}$ (that is, a semiconducting liquid). In previous work aimed at understanding jet formation, theoretical treatments assumed a uniform velocity profile within the Taylor cone and certain authors^{1,3} regarded the cone as an equipotential surface. Here we decided to study the movement of the liquid as it emerges from the capillary and forms the Taylor cone.

To observe the movement of the liquid at the base of the jet, we chose a relatively large capillary, of 1–2 mm diameter, so that the dimensions of the cone were not too small, and tracer particles, lycopodium powder, were added to the liquid. The region of interest was observed and simultaneously photographed at a magnification of about $\times 30$ using a microscope. The experimental set-up consisted of a metal capillary, to which

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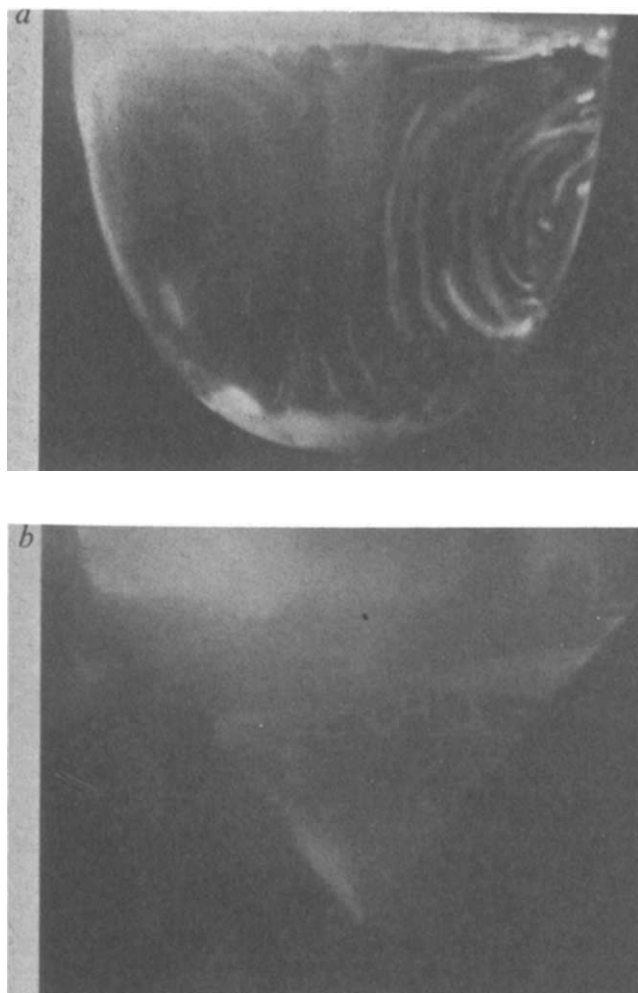


Fig. 2 *a*, Streak photograph of the circulation pattern within an oil drop suspended at the end of a metal rod in helium gas. *b*, Streak photograph of the circulation pattern within the same drop as in *a*, but suspended in air, where a jet is formed.

we applied a high potential directly, and an earthed disk, 5.2 cm in diameter, at a fixed distance of 1.8 cm below the capillary tip. In this set-up the liquid was allowed to impinge on the metal disk. The liquid used was an isoparaffin oil, Isopar M, to which we added *n*-butanol to bring the conductivity within the required limits (see above). The rate of flow of the liquid (8×10^{-11} to $88 \times 10^{-8} \text{ m}^3 \text{ s}^{-1}$) was controlled using a perfusor pump. The jets were illuminated with white light and photographed using a camera.

As the potential was applied and a stable jet was formed, we observed a very clear axisymmetric circulation of the particles in the conical base of the jet. Good streak photographs of this motion were difficult to obtain, but Fig. 1*a* shows this circulation pattern, produced at a flow rate of $8 \times 10^{-9} \text{ m}^3 \text{ s}^{-1}$ (10.5 kV). The direction of motion, which could be identified easily by looking through the microscope, is of great interest. The particles were seen to come down the surface of the cone and reverse upwards along its axis; this was confirmed by taking photographs using a flash light. The light generated by the flash fades after it is triggered so that a moving particle will develop a faint streak in the direction of its motion. Such a photograph is shown in Fig. 1*b*; particles near the axis of the cone have a faint streak pointing upwards. This result is of great significance because it indicates that there is a backflow at the centre whereas the

velocity is greatest at the surface of the liquid. This represents almost the opposite of the velocity distribution for laminar flow in a tube, where the velocity is zero at the wall of the tube and maximum at the centre. This direct observation of circulatory flow invalidates those theories which assume a uniform velocity profile² for the liquid in the Taylor cone.

The origin of this circulation may be understood by reference to a review by Melcher and Taylor⁴ on electrohydrodynamic shear stresses; they pointed out that for an interface to be subjected to a shear stress, the surface must simultaneously support surface charge and be subjected to a tangential electric field. These two conditions are both satisfied in the present system. The electric field within the liquid will drive free charges to the surface of the cone, hence the surface is charged and is subjected to a normal electric field, E_n . Because of the semiconducting nature of the liquid, a potential difference will exist between the base of the cone at the end of the capillary and the tip of the cone. This potential difference ensures that the interface is subjected to a tangential electric field, E_t , in the direction of flow, and hence an electric shear stress on the surface of the cone

$$\tau_t = \epsilon_0 E_n E_t$$

where ϵ_0 is the permittivity of free space. This shear stress causes the axisymmetric convection currents within the Taylor cone that are reported here for the first time.

The velocity profile within the cone was analysed by solving analytically the equations of motion for creeping flow in axisymmetric spherical coordinates. The solution of these equations will be given elsewhere⁵. It is sufficient to mention here that the calculated velocity profiles are consistent with those shown in Fig. 1. Such convection currents were not detected within a water cone, because the high conductivity of water ensures that the cone is almost an equipotential surface. Although the surface is charged it is not subjected to a tangential electric field and shear stress.

Another demonstration of the circulatory motion due to electrohydrodynamic shear stresses was provided by observations of pendant drops inside a glass chamber which could be filled with various gases. We observed that the distortion of a drop hanging from a metal rod connected to a high potential depends on the breakdown potential of the gas. If the breakdown potential is lower than that required to cause instability of the liquid, the atmosphere becomes conducting and no stable jet can be formed. Examples of such gases are He and Ne. For these gases, glow discharges were observed in the dark, and with a semiconducting liquid the flow extended from the edges of the rod down to the earthed plate. The drop was enveloped by the electric discharge. The addition of lycopodium powder to the liquid revealed an axisymmetric circulatory motion but in the opposite direction to that observed within the cone of Fig. 1—that is, near the surface the particles moved upwards and near the centre line of the drop they moved downwards. Figure 2*a* shows the circulation within an oil drop surrounded by He gas.

Again this circulation can be explained as being due to electrical shear effects. The surface of the drop supports free charge due to the field applied, which drives the charges from the bulk to the surface. Hence the first condition for shear stresses to occur is satisfied. The discharge at the edge of the rod where it is in contact with the liquid creates a local potential drop near that region. Hence, the potential at the tip of the drop will be higher than at its edges. This potential difference produces a tangential field and hence a tangential stress across the surface of the drop in the upward direction, and creates the convection currents observed within the drop. If the voltage is increased slightly, a stronger discharge is produced and the motion becomes faster, sometimes causing the drop to climb up the metal rod. If the helium input is stopped and air gradually fills the glass chamber, the discharge disappears and the circulation stops. On further increase in the voltage, the drop develops into

a Taylor cone and from its tip a thin jet is ejected. As soon as this occurs, a circulation pattern is observed (Fig. 2b), the direction of the circulation being the same as that in Fig. 1.

When a pendant drop of a conducting liquid such as water was surrounded by helium, the discharge originated at the surface of the drop itself rather than at the rod, and extended to the earth plate. When tracer particles were introduced into this water drop, no motion of the particles could be observed. Instead, all the particles settled at the bottom of the drop. Again, this result is attributed to the high conductivity of water, which forms an equipotential surface; hence the surface is not subjected to a tangential field.

The above observations clearly demonstrate the role of electrical shear effects in the process of formation of a stable jet. These shear stresses resulting from the electric field within the cone and further down the jet produce a downward longitudinal force which stabilizes the jet by smoothing any perturbations on the surface. As one moves downwards from the cone, the electric field within the jet gradually decreases and so does the shear stress. A point is reached at which the radial force exceeds the shear force, thus causing disruption of the jet into a brush of thin jets which ultimately break into a monodisperse spray; this was confirmed by careful observations of the jet at the point of break-up for semiconducting liquids. With conducting liquids such as water, there is no build-up of electric field within the jet and hence there will be no tangential stress; the jet suffers only the destabilizing effect of the radial stresses and an unstable spray is produced.

The direction of the circular motion within the liquid cone, as demonstrated here, indicates that the jet is formed by the stripping of the drop surface rather than by the pulling of the liquid from the bulk of the drop or the detachment of small droplets from the liquid apex⁶. Thus, it seems that the movement of the thin surface layer of liquid relative to the bulk of the cone under the effect of surface-charge gradients is analogous to the Gibbs-Marangoni effect^{7,8} where the movement of a thin liquid film occurs under the effect of surface-pressure gradients.

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Fractal characterization of inhomogeneous geophysical measuring networks

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The measuring stations of most *in situ* geophysical networks are spatially distributed in a highly inhomogeneous manner, being mainly concentrated on continents and population centres. When inhomogeneity occurs over a wide range of scales in a space of dimension E , it can be characterized by a fractal dimension D_m . For measuring networks, there is no reason to assume *a priori* that D_m equals E ; it will usually be less than E . The world meteorological network studied here is an example on a surface for which $E = 2$ (the surface of the Earth) whereas, the network has an



Fig. 1 The locations of the 9,563 stations in the global meteorological measuring network (defined as the stations which the World Meteorological Organisation lists as performing at least one meteorological measurement per day), showing their high degree of non-uniformity. Most are clustered on the continents and major industrial areas. The dimension (D_m) is ~ 1.75 .

empirical dimension $D_m \approx 1.75$. Whenever $D_m < E$, any sufficiently sparsely distributed phenomena (with dimension $D_p < E - D_m$, here 0.25), cannot be detected—even if the network is infinite. Because these rare phenomena are the most intense, this insufficient dimensional resolution is associated with biases in geophysical statistics, serious difficulties in interpolating measurements to a uniform grid, and problems in calibrating remotely-sensed information.

The intuitive notion of dimension D_m of a set (whether fractal or otherwise) is given by the variation of the number of points ($\langle n(L) \rangle$) with the size of the region (L):

$$\langle n(L) \rangle \propto L^{D_m}$$

When D_m is less than the dimension (E) of the space in which the set is embedded, when L increases, the volume of space available increases as L^E which is faster than the number of points of the set that are available to fill it ($\langle n(L) \rangle$). Hence, larger and larger 'holes' (empty regions) appear and the set is concentrated on a decreasing fraction of the total space. Furthermore, at a given scale, sets with lower D_m are more dominated by holes, and are hence more sparse.

Although there are many ways of estimating the dimension of sets of points, many of which have been developed for studying strange attractors^{2–5} they generally yield similar results. The method used here, actually determines a particular dimension called the correlation dimension⁶. To apply this method to a geophysical network (considered here as a set of points), determine, for each station in the network, the number ($n(L)$) of other stations within various radii L of the point, and its average $\langle n(L) \rangle$ over all the stations. We then obtain D_m as the slope of $\log \langle n(L) \rangle$ against $\log L$. Scales over which $\langle n(L) \rangle$ varies non-algebraically, define the characteristic lengths of the network.

To apply this technique to a surface network (such as the highly clustered world meteorological network, Fig. 1), we must discuss a complication that takes into account the curvature of the Earth's surface. If the latter is covered uniformly with stations in a region of area S , then $\langle n(L) \rangle \propto S$. Taking $S(\theta)$ as the area of the spherical cap defined by two points subtending an angle θ at the Earth's centre (radius r) we may define the corresponding scale $L(\theta)$ by:

$$S(\theta) = (\pi/4)L^2(\theta) = 2\pi r^2(1 - \cos \theta/2)$$

Note that with this definition of L , for small θ , the formula reduces to the usual great circle distance ($= r\theta$), and we recover $D_m = 2$ when the stations are homogeneously distributed.

Figure 2 shows $\langle n(L) \rangle$ calculated using the above L , for the 9,563 stations in this network separated by at least 0.01° of arc

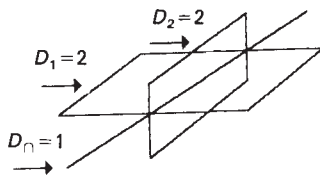
(~ 1 km, the accuracy of the data). Avoiding double counting, each station defines $9,562/2 = 4,781$ values of L , hence there are $9,563 \times 4,781 = 45,720,703$ independent values that go into the histogram for estimating $\langle n(L) \rangle$. Figure 2 shows that roughly between the minimum resolvable scale, (~ 1 km) and $\sim 2,000$ km, $\langle n(L) \rangle \propto L^{1.75}$, hence $D_m \sim 1.75$. The size of the scaling regime is limited as the number of points is finite: $\langle n(L) \rangle_{\max} = 4,781$ and $\langle n(L) \rangle_{\min} \sim 1/9,563 \sim 10^{-4}$. Assuming $L^{1.75}$ between these limits implies $L_{\max} \sim 7,500$ km, $L_{\min} \sim 0.3$ km, which shows that the scaling observed in Fig. 2 spans a range nearly as large as is possible. Similar analysis of the French climatological network (3,593) stations yielded $D_m \sim 1.8$, while the Canadian meteorological network (414 stations) yielded $D_m \sim 1.5$.

The fact that $D_m < E$, sets new detectability limits. A network, dimension D_m can detect a phenomenon with dimension D_p only if the two sets intersect. However, a theorem in geometry shows that this is certain to occur only when $D_p > E - D_m$. Two sets, dimension $= D_1, D_2$ embedded in a space dimension $= E$, intersect on a set dimension $= D_{\cap}$. (With the co-dimension $C = E - D$) according to the following rule:

$$C_{\cap} = \text{Inf}((C_1 + C_2), E)$$

Example. Intersection in space ($E = 3$) of two planes

$$(D_1 = D_2 = 2 \Rightarrow C_1 = C_2 = 1)$$



$$C_{\cap} = C_1 + C_2 = 2 \Rightarrow D_{\cap} = E - C_1 = 1$$

Although the example is for two standard sets (planes) it holds for most fractal sets. Note that if $C_m + C_p > E$, the intersection set probably has dimension zero ($C_{\cap} = E$), hence, the sets typically miss each other. Hence, in the case studied here, sparse surface phenomena with $D_p < 2 - 1.75 = 0.25$, cannot be detected. In analogy with the network's spatial resolution, which is the minimum detectable scale, we may define, its dimensional resolution as $E - D_m$ which is the minimum resolvable dimension.

The inability to detect sparse phenomena is serious because, in general, we expect geophysical fields to be characterized by multiple fractal dimensions⁶⁻⁹. In turbulence, fields are generally characterized by multiple fractal dimensions¹⁰⁻¹³. In geophysics, there is empirical evidence for multidimensionality in the rainfall^{14,15}. If a field is multidimensional, then regions exceeding a threshold T define fractal sets with dimension $D_p(T)$ decreasing with T . Thus, whenever averages are taken over sets with $D_m < E$, then, by the intersection theorem, the most intense fractals (with $D_p(T) < E - D_m$) are missed. While both mono- and multidimensional fields have statistical properties that are functions of scale, multidimensional fields are in addition dependent on the dimension (for example, line, plane, volume or fractal set) over which they are averaged.

We have argued that the characterization of network inhomogeneity by the fractal dimension (D_m) raises new problems concerning the detectability of sparse phenomena. Since phenomena (of any size) with $D_p < E - D_m$ will not intersect the network, we have obtained a new criterion for evaluating measuring networks: to detect phenomena, not only must a network have sufficient spatial resolution it must also have sufficient dimensional resolution. In general, we expect the fractal dimension of geophysical fields to be a decreasing func-

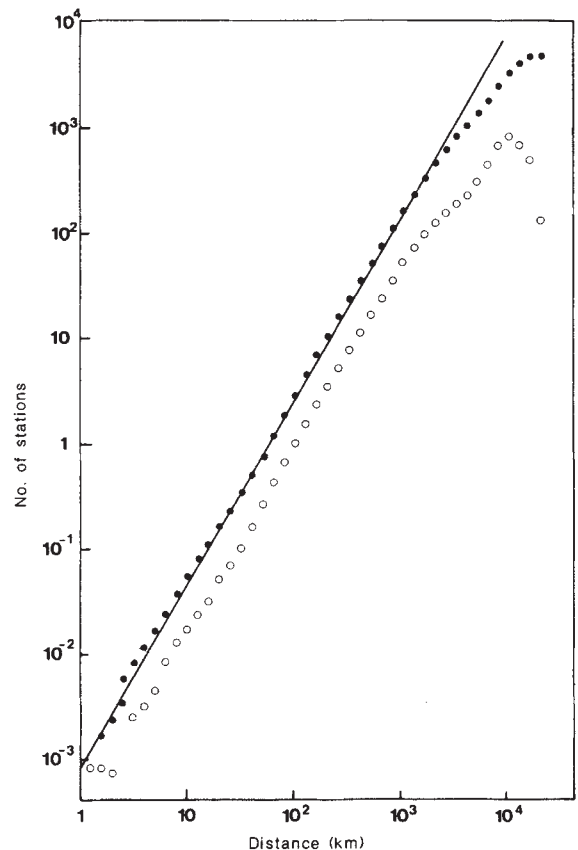


Fig. 2 ○, The average number of stations within annuli of geometrically increasing radii; ● (the integral of the previous function), the function $\langle n(L) \rangle$ described in the text. Over the scaling regime, both should be parallel and straight: the $L^{1.75}$ function is shown for reference. The scaling regime apparently continues down to scales comparable with the accuracy of the geographical locations used to determine L (~ 1 km) which is, therefore, the spatial resolution of the network. For comparison, the standard analysis method (which assumes $D_m = 2$, a surface area of $\sim 10^8$ km², and 10^4 stations), attributes an average area of $10^8/10^4 = 10^4$ km² per station hence a spatial resolution of $\sim 10^2$ km² which is about 100 times larger than its true resolution.

tion of intensity: thus, any lack of dimensional resolution leads to biases in the spatial averages. Finally, as information on the lowest dimensional fractals is lost, similar biases will arise when measurements are interpolated to uniform grids with dimension E .

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Archaean magmatic sulphate

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The absence of redbeds, the occurrence of readily oxidizable minerals in placers, and the rarity of sulphate minerals are some features which suggest generally reduced conditions on the Earth's surface during Archaean time¹. Supporting this conjecture are $\delta^{34}\text{S}$ values near 0‰ for sulphides of sedimentary rocks²⁻⁴ and massive sulphide deposits⁵, since isotopic fractionation requires oxidation-reduction reactions. The flux of oxidized species during the Archaean produced by fixation of organic carbon was, however, comparable with modern levels⁶, but was largely consumed by reactions with a large supply of reduced mantle material^{4,7}. Notable occurrences of Archaean sulphate are ~3,400-Myr stratiform barites in Australia^{8,9}, South Africa¹⁰ and India¹¹, with $\delta^{34}\text{S}$ ~+4‰ (refs 8-10) which are believed to have formed by bacterial oxidation of primary (0‰) sulphide¹⁰. Consideration of the Archaean sulphur cycle has focused on sulphate of this origin. We draw attention here to sulphate of different isotopic composition, +8 to +14‰, of magmatic origin, that contributed to the total flux of oxidized species during the Archaean.

Occurrences of sulphate minerals in Archaean terrane (Table 1) have been divided into vein and stratiform types. These are mainly barite or anhydrite. Where gypsum is found, it appears to have formed by hydration of primary anhydrite at low temperatures. It is important to establish first that these sulphates are of Archaean age and were not introduced by later processes. We have used Sr isotope determinations for this purpose. As both barite and anhydrite generally contain >1,000 p.p.m. (parts per 10⁶) Sr and negligible Rb, measured and initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are the same. Most samples are of late Archaean age (2,800-2,600 Myr) and have ratios in the range 0.7013-0.7021 (Table 1). This is comparable with initial ratios of magmatic rocks of this age, for example, 0.7014 to 0.7018 for mafic volcanic rocks from the Abitibi belt, Canada¹² and 0.7013 for mafic volcanic rocks from Kambalda, Australia¹³. Values for felsic intrusives are in a similar range^{14,15} except for higher ratios for intrusives derived by partial melting of pre-existing rock¹⁶.

The low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of sulphate minerals are taken to indicate that these sulphate minerals formed during volcanism or during the following plutonism and metamorphism of the belts. Because of the secular increase in the Sr isotopic composition of ocean water¹⁷ and igneous rocks¹⁴, their formation at a later time, that is, in the Proterozoic, from either of these sources, would produce higher $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. Furthermore, solutions passing through Archaean rocks at a later time may gain ^{87}Sr produced by decay of ^{87}Rb . Based on their Sr isotope values, we have eliminated some occurrences within Archaean rocks, for example, barite veins in volcanic rocks in the Thunder Bay district with values >0.710 (ref. 18) and barite veins in the Timmins-Matachewan area with ratios of 0.7030 to 0.7050 (ref. 18). In both cases, some of the veins of similar occurrences in the areas cut Proterozoic rocks, confirming their post-Archaean origin.

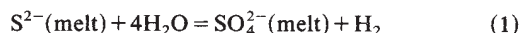
Most of the vein occurrences referenced in Table 1 are associated with gold mineralization. The time relationship of the sulphate veins with gold mineralization varies from pre-gold and penecontemporaneous with the host intrusive (McIntyre Mine¹⁹); contemporaneous with the gold mineralization (Golden Mile, Kalgoorlie (J. M. F. Clout and E.M.C., unpub-

lished data), Lac Shortt²⁰); to post-gold (Lake Shore and Macassa Mines²¹). At Lake Shore and Macassa in Kirkland Lake the sulphates occur in shear zones. Since mineralization and igneous activity took place during the Archaean, the associated sulphate also probably formed at this time. The continuous movement of the shear zones during the late Archaean and Proterozoic may explain their high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (Table 1), by incorporation of radiogenic Sr.

Reported occurrences of stratiform barite have been from older ($\geq 3,000$ Myr) Archaean rocks. Until recently there was no known occurrence in Archaean strata of North America, which are mostly 3,000-2,670 Myr (ref. 22). Elsewhere, the only barites that may be stratiform and late Archaean are in the Bulawayan Group of Zimbabwe, but their genesis and age are uncertain²³. In 1981, the large Hemlo gold deposit, near Marathon, Ontario, was discovered. The tabular ore bodies appear to be stratiform near the facies contact between volcanoclastic and epiclastic sedimentary rocks. The main ore zone and footwall rocks contain massive barite layers and lenses up to 10 m in thickness²⁴. More recently, massive barite bands, which are discontinuous but on strike >6 km within a metasedimentary sequence, have been found 21-26 km west from the Hemlo mineralization²⁵.

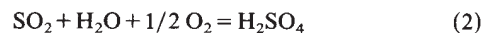
Whereas $\delta^{34}\text{S}$ values for the Barberton and North Pole barites are ~+4‰, most of the other sulphate minerals are in a range of +7 to +14‰. This is too large to be explained by simple oxidation of primary magmatic sulphide. Sulphates of this composition could only have formed by two processes: ^{34}S -enrichment of biologically-produced sulphate of ~4‰ by its partial reduction to ^{34}S -depleted sulphide; or formation by magmatic or magmatic-hydrothermal processes with $\delta^{34}\text{S}$ values being determined by inorganic isotopic exchange reactions at high temperature.

Sulphate in igneous rocks is formed by two processes: oxidation of primary sulphide in silicate magma (magmatic sulphate) and hydrolysis of SO_2 gas in aqueous fluids (magmatic-hydrothermal sulphate). The solubility of sulphur in silicate melts depends on oxygen fugacity (f_{O_2}) as well as temperature and pressure²⁶, and the contents usually range from ~800 to ~1,100 p.p.m. in mafic volcanic rocks²⁷⁻²⁹. Felsic magmas may have contained more³⁰, although they tend to lose most of the sulphur during intrusion and solidification. Most of this sulphur is present as sulphide and sulphate with the relative proportions controlled by f_{O_2} (ref. 26). The f_{O_2} is determined by the mineral assemblages^{26,28}, principally the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratios, as the amount of iron is far larger than that of sulphur. Overall reaction for the oxidation of sulphur in silicate magma is expressed as²⁸



Evidence for this process is a correlation between the water and sulphate contents in basaltic glass²⁸ and an increase in δD values of water in pillowed basalt due to degassing of low δD gas, such as CH_4 and/or H_2 (ref. 31). Selective degassing of H_2 from magma causes reaction (1) to move to the right, oxidizing the magma²⁸. Such magmatic sulphate comprises 15-36% of the total sulphur^{28,29}. The magmatic sulphate is retained in the glass phase on solidification.

In a more open system, such as subaerial extrusives, much of the sulphur is lost as gas, SO_2 (refs 29, 32), reducing the magmatic sulphate content in the remaining silicate melt, often to <30 p.p.m., and f_{O_2} . The escaping SO_2 gas from magma is eventually hydrated to SO_4 in the atmosphere by the following reactions, even in low p_{O_2} (partial pressure of O_2) conditions:



SO_2 gas dissolved in an aqueous fluid in igneous rocks at high temperatures will hydrolyse to form sulphate in the magmatic-hydrothermal system³³:



Table 1 Sulphate minerals in Archaean greenstone belts

Locality	Mineral*	Age†	Occurrence‡	Mineralization§	⁸⁷ Sr/ ⁸⁶ Sr	δ ³⁴ S _{CD} ‰
Massive stratiform						
Barberton, South Africa	BR	~3.4	Some are replacement of GY ⁴⁶		0.7009 to 0.7017 (<i>N</i> = 8) ¹⁰	av. +3.4 ± 0.3 (<i>N</i> = 7) ¹⁰ +3.6 to +4.9 (<i>N</i> = 6) ⁸
North Pole, Western Australia	BR	~3.4	Some are replacement of GY ⁹		0.7004 (<i>N</i> = 2) ⁸	av. +3.6 ± 0.5 (<i>N</i> = 32) ⁹ +11, +13, +13 ⁹
Big Stubby, Western Australia	BR	~3.4	In felsic volcanics ⁵²	Cu, Zn, Pb		
Bien Venue, South Africa	BR	~3.1	In felsic volcanics	Cu, Zn, Pb	0.7011 (<i>N</i> = 1)‡‡	+3 to +13 (<i>N</i> = 4)‡‡
Hemlo, Ontario	BR	~2.7	Base of Au mineralization and in footwall rocks	Au, Mo	0.7017 to 0.7018 (<i>N</i> = 5) ²⁴	+8 to +12 (<i>N</i> = 40) ²⁴ ¶
Northern Eagle, Padre, Kadrey, Ontario	BR	~2.7	West and on strike from Hemlo	(Mo, As)††	0.7017 to 0.702 (<i>N</i> = 3) ²⁴	+2, +8, +10‡‡
Stratiform disseminated						
Lac Shortt, Quebec	BR	~2.6	Minor amount in ore ²⁰	Au		
Stratabound disseminated or discontinuous lenses						
Hemlo, Ontario	AN	~2.7	Disseminated or small lens in footwall rocks	Au	0.7013 ²⁵	+8 (<i>N</i> = 3)‡‡
Monomineralic vein (lens)						
McIntyre, Ontario	AN	~2.6	In Q-F-P ¹⁹	Au, Cu	0.7011‡‡	+8, +10‡‡
Lake Shore, Ontario	BR	~2.6	In syenite	Au	0.7048, 0.7056‡‡	+12, +13, +14, +14‡‡
Macassa, Ontario	AN	~2.6	In ore vein in syenite ²¹	Au	0.7073, 0.7078‡‡	+14‡‡
	BR		In syenite ²¹			
Lamaque, Quebec	AN	~2.6	In Q-F-P ⁴⁷	Au		+13 to +14 ⁴⁷
Young-Davidson, Ontario	BR	~2.6	In syenite ⁴⁸	Au		
Ross, Ontario	AN	~2.6	In andesite, dacite, syenite	Au	0.7023 (<i>N</i> = 2)‡‡	+8, +9, +10‡‡
	BR		In ore veins ²¹			
Golden Mile, Western Australia	AN	~2.7	In granophyre unit of Golden Mile Dolerite	Au	0.7015 (<i>N</i> = 2)¶ (J. H. F. Clout and E.M.C. unpublished)	+12 (<i>N</i> = 2)¶
Eye-Dashwa Lake, Ontario	GY	~2.7	In granite ^{49,50}		0.7045 (<i>N</i> = 1) ⁴⁹	av. +7.1 ± 0.4 (<i>N</i> = 8) ⁴⁹ +7.3, +8.2 ⁵⁰
Lac du Bonnet, Manitoba	GY	~2.7	In granite ⁵⁰			
Others not well known						
Con Mine, Northwest Territories	AN	~3.1**	Disseminated in tonalite boulder in shear zone ⁵¹	Au		+6.8, +7.0, +7.2 ⁵¹
Heron Bay, Ontario	AN BR	~2.7	Monomineralic lens in volcanics	Au, Mo	0.7021 (<i>N</i> = 2)‡‡	+9‡‡

* BR, barite; AN, anhydrite; GY, gypsum.

† Age of the host rocks.

‡ Q-F-P; quartz feldspar porphyry; vol, volcanic rocks; sed, sedimentary rocks.

§ Precious and base metal mineralizations in the area.

|| The minimum value was listed for anhydrite. See text for explanation.

¶ Values for the massive basal barite.

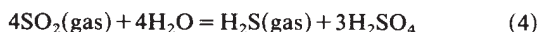
The data are taken for anhydrite closely associated with gold mineralization since late stage sulphates have higher values.

** The ²⁰⁷Pb/²⁰⁵Pb age of zircon in the boulder containing anhydrite.

†† Minor amounts associated with the massive barite.

‡‡ This study.

Diffusive loss of H₂ gas will move the reaction to the right. At temperatures below the H₂O-saturated solidus, hydrolysis of SO₂ can also yield H₂S gas and sulphate³³:



Precipitation of the sulphate by reaction with Ca²⁺ to form anhydrite in aqueous fluid moves the reaction to right.

Epizonal intrusives are particularly suitable for developing sulphate of this latter origin. The low lithostatic pressure facilitates the exsolution of H₂O and S from silicate magmas; brecciation due to the secondary boiling, which allows escape of gas phases; and ready access to surface water. The occurrence of sulphate minerals in the hydrothermal system of porphyry copper mineralization is well documented^{34,35}.

The sulphur isotopic composition of magmatic sulphate will depend on the temperature, the ratio S²⁻/SO₄²⁻, and the primary S isotopic compositions of the magma, which is ~0‰ for Archaean magmas because of lack of contamination of sedimentary rocks containing sulphide of δ³⁴S different from 0‰. The isotopic fractionation factor, α, in melts³⁶ at 600–1,000 °C is:

$$1,000 \ln(\alpha_{\text{SO}_4^{2-}-\text{S}^{2-}}) = 7.4 \times 10^6 / T^2 - 0.19$$

where *T* is in K. α_{SO₄²⁻-S²⁻} is 5‰ at 900 °C, 8‰ at 700 °C and 9‰ at 600 °C. There may be re-equilibration in silicate glass during cooling after solidification, giving lower apparent formation temperatures²⁸. Because sulphide is dominant in most magmas, it will show the least departure from the primary isotopic composition of 0‰, with sulphate being in the range of +7 to +12‰ (refs 28, 29). However, degassing of H₂ can increase the sulphate content, causing δ³⁴S for the latter to decline, for example, Hawaiian subaerial flows²⁹. δ³⁴S for sulphate in magmatic-hydrothermal fluids are larger than that of magmatic sulphate because of lower temperatures of the fluids compared with melts.

When δ³⁴S for total S is ~0‰, δ³⁴S values of magmatic sulphate will mostly be in a range of +7 to +10‰ for mafic rocks formed at ~1,000 °C and higher, to ~+12‰ in felsic rocks with lower solidification temperatures. The values of magmatic-hydrothermal sulphate formed in aqueous fluids is mostly in the range of +8 to +14‰ (refs 35, 37). An important point is that the rate of formation of magmatic and magmatic-hydrothermal sulphate is independent of the atmospheric oxygen pressure. It is dependent on the intensity of volcanism over a given time and the tectonic conditions in which this takes place.

For the vein sulphates listed in Table 1, which are mostly associated with intrusions and which have $\delta^{34}\text{S}$ values of +8 to +13‰, it is reasonable to suggest that these are of magmatic-hydrothermal origin. They are analogous to the veins of anhydrite on Phanerozoic porphyry mineralization. What is less certain is the origin of the +8 to +13‰, tabular sulphate body parallel to bedding, such as that at the Hemlo main deposit and barite occurrences west of Hemlo. These appear to have precipitated by the interaction of Ba^{2+} -rich fluid and sulphate-rich water²⁴. This requires the earlier accumulation of sulphate in the surface water since sulphate was generally poor in the Archaean, both in continental and submarine environments.

There are two possible origins for this sulphate: (1) biological oxidation of primary sulphide to form ~0 to ~+5‰ sulphate in a basin, followed by (1a) biological or (1b) abiological enrichment in ^{34}S ; or (2) introduction of magmatic and/or magmatic-hydrothermal sulphate. In both cases, there is a requirement that the basin be restricted to prevent its dissipation within the sulphate-poor Archaean ocean waters⁴.

Option (1a) can be largely disregarded since there is a general lack of pyritic graphitic shale in the footwall rocks of the Hemlo succession. Six samples of pyrite from the limited occurrences of graphitic shale have $\delta^{34}\text{S}$ values ranging from +1.8 to +3.5‰. It is not possible to identify whether options (1b) or (2) was dominant. However, a magmatic or magmatic-hydrothermal contribution of sulphate from high-level magma seems possible, considering the intense volcanism in the region³⁸; the abundance of high level intrusives, including porphyries; the essential lack of sedimentary rocks in the footwall; and the overall association between sulphates and gold mineralization demonstrated above.

How important might the flux of magmatic and magmatic-hydrothermal sulphate be during the Archaean? The oxidation condition of magmas is thought to have been similar since Archaean time, judging from similar mineral assemblages³⁹. Assuming ~900 p.p.m. S in magmas, of which 20% is sulphate, an evaluation may be made of the production rate of magmatic sulphate. At present, the total output of volcanic rocks is estimated to be ~26 to ~34 km³ yr⁻¹, 23 km³ yr⁻¹ in the oceanic environment and 3–10 km³ yr⁻¹ in the continental environment⁴⁰. Using these values, the total amount of sulphate presently produced by submarine magma can be calculated: 23×10^{15} (volume of rocks) $\times$ 2.4 (specific gravity) $\times$ 900 $\times 10^{-6}$ (content of S) $\times$ 0.2 (proportion of SO_4^{2-}) $\times$ 96/32 (conversion from S to SO_4^{2-}) = $\sim 3 \times 10^{13}$ g yr⁻¹. The formation of SO_4 in continental environments is difficult to evaluate because the high rate of

SO_2 production from present-day volcanoes, $1.2 \times 96/64 \times 10^{13}$ g = 1.8×10^{13} g (ref. 41) may reflect contamination and assimilation of magma by sulphur-rich sedimentary rocks and because the size of continents in Archaean time is uncertain⁴². If we assume that continental volcanism has been similar throughout geological time, the total production of magmatic sulphate is calculated to be 4.8×10^{13} g yr⁻¹. This compares with $\sim 1.8 \times 10^{14}$ g yr⁻¹ produced by weathering processes⁴³, so that ~27% of the annual production is magmatic sulphate. Even if we ignore the continental supply, the magmatic sulphate would constitute ~17%.

If magmatic and magmatic-hydrothermal sulphate were to accumulate in the Archaean ocean, the present concentration in sea water, 4×10^{21} g (ref. 43), would have been reached in ~100 Myr. The quantity estimated is a minimum, since volcanism may have been several times more intense at 2,700 Myr (ref. 44) and an undefined quantity of magmatic-hydrothermal sulphate must be added. It can thus be concluded that there was a significant contribution of the magmatic sulphate to the flux of oxidized species in the surface environments in the Archaean and, indeed, in later periods.

Intense volcanism and fast seafloor spreading during the Archaean^{42,45} provided a large quantity of reduced mantle material to the Earth's surface. Because of the high rate of interaction between the reduced iron and manganese from the mantle and the hydrosphere at the spreading ridges and subduction zones, the oxidized species formed in the hydrosphere were removed from the Archaean ocean^{4,7}. The prevailing reduced conditions in the Earth's surface both in continental and submarine environments was, thus, maintained in Archaean time, and evidence for the presence of sulphate in the hydrosphere, therefore, will be generally lacking. Evidence of magmatic sulphate will only be retained locally in rocks which escaped subduction, such as back-arc basins and rift systems in continents. Preserved continental magmatic-hydrothermal systems, as described above, are other examples.

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Carbon isotope excursions in Precambrian/Cambrian boundary beds, Morocco

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The Precambrian/Cambrian boundary is one of the most significant points on the geological timescale, because it marks the change in the fossil record from soft-bodied organisms to skeletonized faunas. The Cambrian explosion or radiation event¹ saw the evolution of many new life forms, especially various molluscs and archaeocyathids from the base of the Cambrian (Tommotian stage). Others, including the trilobites and bivalves, evolved a little later in the succeeding Atdabanian stage^{2,3}. A massive increase in the biomass of the photic zone through phytoplankton blooms has also been postulated for this time⁴, particularly in the late Riphean/early Vendian and in the early Cambrian⁵. There has been much discussion concerning where precisely to place the boundary^{6,7} and there are several contenders in the world for the type section⁸. I present here a carbon isotope profile, from the Precambrian/Cambrian boundary beds in Morocco, where two distinctive positive $\delta^{13}\text{C}$ excursions are revealed. These are interpreted as records of increased organic productivity as part of the Precambrian/Cambrian explosion of life. $\delta^{13}\text{C}$ excursions could be useful points for correlation between boundary sections.

The late Precambrian/Cambrian strata are extensively exposed on the northern slopes of the Anti-Atlas mountains in southern Morocco⁹. The 'classic' section occurs near the village of Tiout, 25 km south-east of Taroudannt, 80 km east of Agadir (Fig. 1). The sequence begins with the Dolomie Inferieur, 200 m of dolomite with local cryptalgal, grainstone and replaced evaporite horizons. The succeeding Serie Lie de Vin (950 m) consists of cyclically arranged limestones, dolomites and fine clastics which represent rhythmic fluctuations in the position of the shoreline¹⁰. Algal thrombolites and stromatolites occur throughout the sequence and include the genera *Tungussia*, *Patomia* and *Nimbophyton*^{11,12}. The Calcaire Superieur (220 m) consists of massive limestones (some black, some oolitic), dolostones, chert nodules and the stromatolite *Acaciella*. The first trilobites (small opisthoparians¹³) appear towards the top of the Calcaire Superieur, and within a few metres there are archaeocyathid bioherms¹⁴. The upper, fossiliferous 130 m of the Calcaire Superieur have been separated off to form the Transition Beds^{10,12} (Fig. 2). Shales with the trilobite *Fallotaspis* mark the beginning of the Serie Schisto-Calcaire¹³, which consists of many transgressive oolite-shale cycles¹⁵. Overall, the Moroccan sequence reflects mostly shallow-water shelf sedimentation on the subsiding north-west margin of the Saharan Craton, adjacent to the opening Iapetus (Proto-Atlantic) Ocean¹⁶.

There has been much discussion on the position of the Precambrian/Cambrian boundary at Tiout, as at other boundary candidates, but there now appears to be a consensus. On the basis of a detailed study of the stromatolites, notoriously difficult to use for dating, the Serie Lie de Vin is placed in the Vendian (latest Precambrian)¹¹. An alternative view on the stromatolites suggests that they are lowest Cambrian¹⁷ but a similar Vendian age for the Lie de Vin is derived from acritarchs¹⁸. The archaeocyathids indicate a mid-Atdabanian age for the Transition Beds¹⁴, suggesting that the Calcaire Superieur is lower Atdabanian and Tommotian. The Tommotian is recognized by the occurrence of small shelly fossils¹, which are mostly phosphatic, but unfortunately these have not yet been found in the Anti-Atlas despite acid digestion of many samples¹¹. The Tommotian stage was generally a regressive time² so that its deposits

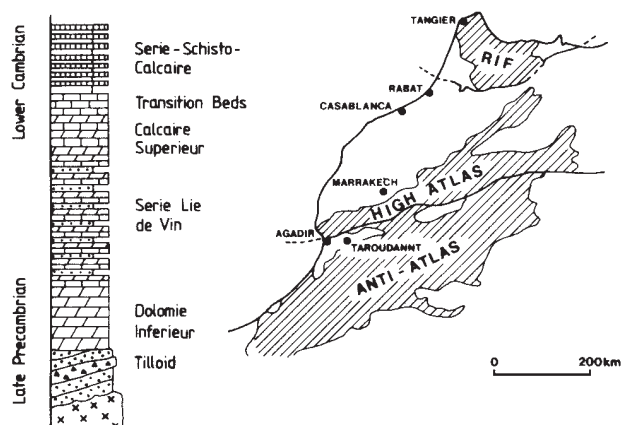


Fig. 1 Location map of Morocco, showing the three principal tectonic units, the Anti-Atlas, High Atlas and Rif, and the stratigraphy of the late Precambrian/Cambrian beds in the Anti-Atlas.

may be thin or even absent. The Precambrian/Cambrian boundary is thus likely to occur in the Lower Calcaire Superieur or Upper Serie Lie de Vin. The Dolomie Inferieur is attributed to the late Riphean^{10,18}. A tilloid of probable glacial origin occurs in the Tiddiline Formation, unconformably beneath the Dolomie Inferieur¹⁹.

The results of whole-rock, carbon stable-isotope analyses of limestones and dolomites from the Anti-Atlas boundary beds are presented in Fig. 2. With such determinations, there is always the possibility that the values have a diagenetic component through cement precipitation, calcite neomorphism or dolomitization, so that the values do not reflect the isotopic composition of contemporaneous sea water. However, it is generally held that the marine carbon-isotope signature is less prone to alteration during diagenesis than the oxygen isotope as the reservoir of carbon is very much smaller than that of oxygen in diagenetic porewaters²⁰. When recrystallization and dolomitization of calcite or aragonite grains and muds take place, in most cases the carbon-isotope ratio is altered little because the carbon atoms are almost entirely derived from the original carbonate.

In organic-rich sediments, diagenetic calcites and dolomites can be very light to quite heavy (-20 to $+10\%$ $\delta^{13}\text{C}$), as a result of the various diagenetic breakdown processes affecting organic matter²¹. However, organic-rich carbonates were not analysed in this study, and in fact, other late Precambrian organic-rich limestones (from Norway²² and California; unpublished data) analysed have typical marine $\delta^{13}\text{C}$ values (average 1.7 and 2.0‰ respectively). Meteoric waters with soil-derived CO_2 can give extreme $\delta^{13}\text{C}$ signatures to carbonate precipitates, but these are typically negative, rather than positive, and can be expected to relate to prominent emergence horizons (absent in the Precambrian/Cambrian of the Anti-Atlas). Finally, there is little isotopic fractionation of carbon isotopes with temperature.

The profile in Fig. 2 is derived from limestones and dolomites from a variety of open, shallow-marine facies and degrees of diagenesis, but the broad pattern of $\delta^{13}\text{C}$ change is considered to be a genuine record of changes in $\delta^{13}\text{C}$ of the original marine precipitates. As an example, there is no significant difference between the $\delta^{13}\text{C}$ values of oolites and stromatolites in the Serie Schisto-Calcaire, or between bioclastic and oolitic limestones in the Transition Beds at the top of the Calcaire Superieur. The oxygen-isotope data, not presented here, show more scatter; this is expected as diagenesis can affect the $\delta^{18}\text{O}$ considerably, mainly through the precipitation of ^{18}O -depleted calcite spar cement²³, and there can be a several per mil difference between limestones and dolomites. The validity of the very positive $\delta^{13}\text{C}$ values of the Dolomie Inferieur might be questioned but, in fact, their oxygen-isotope values (M.E.T., unpublished data) are

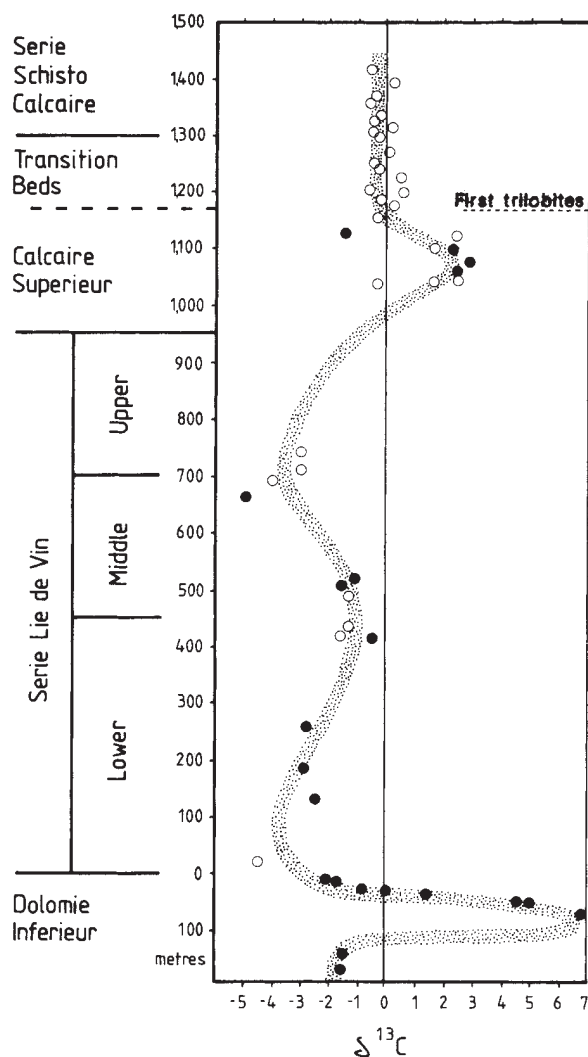


Fig. 2 Carbon-isotope stratigraphy from the late Precambrian/Cambrian boundary beds, Tiout, near Taroudannt, Anti-Atlas. Closed symbols are dolomites; open symbols are limestones.

in the same range (-6 to -9% PDB) as the samples above and below, suggesting no major difference in the diagenesis. This deduction is supported by microscopic studies. Thus, the $\delta^{13}\text{C}$ isotope profile of Fig. 2 is considered to be a reflection of changes in the carbon-isotopic composition of sea water during the critical late Precambrian/Cambrian time.

The carbon-isotope profile shows two positive excursions in $\delta^{13}\text{C}$, one near the top of the Dolomie Inferieur and the other within the Calcaire Supérieur. The Serie Lie de Vin has a moderately negative signature, and the limestones of the Transition Beds and Serie Schisto-Calcaire are close to 0% PDB. The carbon-isotope signature of marine limestones (and many dolomites) tends to reflect the $\delta^{13}\text{C}$ of total dissolved inorganic carbon (TDC) in sea water. Average oceanic $\delta^{13}\text{C}$ TDC today is $\sim 0\%$ and shallow marine carbonate precipitates are in the range 0 to 4% PDB (refs 20, 23). Organic carbon, on the other hand, has very negative $\delta^{13}\text{C}$, in the range -20 to -30% , since the lighter ^{12}C is preferentially incorporated into organic matter. It is generally assumed that the input of carbon to the oceans has remained relatively constant through time and that the ocean carbon reservoir mass had changed little. Changes in oceanic $\delta^{13}\text{C}$ TDC are thus thought to be caused by variations in the carbon output to the organic carbon (C_{org}) and carbonate carbon (C_{carb}) reservoirs^{24,25}. At present, a $C_{\text{org}}/C_{\text{carb}}$ output ratio of

around $1:4$ maintains the steady-state $\delta^{13}\text{C}$ of $\sim 0\%$ in oceanic water. Increases in the burial rate of the ^{12}C -enriched organic carbon, relative to the amount of carbonate carbon being precipitated, thus lead to a positive or heavier isotopic composition of seawater TDC, and of carbonate precipitates therefrom. Higher rates of organic carbon burial could result from increases in organic productivity. Alternatively, they could reflect an increase in the preservation potential of the organic carbon; this could result from oceanographic effects, such as the development or expansion of oxygen-depleted oceanic water masses. Positive excursions of $\delta^{13}\text{C}$ have been detected in Cretaceous and Jurassic limestones^{26,27}, and correlate with oceanic anoxic events (OAEs), when increased organic productivity through eustatic sea-level rises leads to the burial of much organic matter and depletion of the oceanic TDC in ^{12}C .

The two positive $\delta^{13}\text{C}$ excursions revealed in the Morocco boundary beds can be interpreted as indicating increased levels of organic productivity, resulting in the preferential extraction of ^{12}C from the oceanic carbonate reservoir. The excursion at the top of the Dolomie Inferieur is probably close to the Riphean/Vendian boundary, a time of radiation of eukaryotic plankton⁵; it could reflect a major increase in the microplankton in the oceans, perhaps an evolutionary event related to a post-glacial climatic warming or more vigorous oceanic circulation connected with the opening of Iapetus, which took place around this time¹⁶. There are no major facies changes through the peritidal Dolomie Inferieur, so that the $\delta^{13}\text{C}$ event is unlikely to reflect increased productivity through a eustatic sea-level rise, as is generally the case with the Cretaceous OAEs²⁸. The Dolomie Inferieur is not particularly organic-rich but the organic matter would probably be buried in deeper water sediments of contemporaneous basins and oceans. Such organic-rich deposits occur in the early Vendian of the Sparagmite Basin, south Norway²². The second positive excursion, in the Calcaire Supérieur, is not so extreme but occurs in strata probably containing the Tommotian/Atdabanian boundary. This was a time of rapid metazoan evolution^{1,2} and a second radiation event of the microplankton⁵. These increased levels of organic productivity can be expected to have affected the oceanic carbon budget. After each $\delta^{13}\text{C}$ excursion, the sea water returned to more normal values as recycling of the organic matter restored the balance of the organic carbon/carbonate carbon reservoirs.

The late Precambrian/early Cambrian was a time of oceanic chemical events. An extreme rise in the sulphur-isotope ($\delta^{34}\text{S}$) curve has been termed the Yudomski Event²⁹ and attributed to the catastrophic mixing of brine in local basins with the surface ocean water. An OAE and a phosphogenic event have been postulated for this time⁴, along with catastrophic mixing of deep anoxic waters with aerated surface waters^{4,30}. These chemical events have been invoked as major factors in organic evolution (as well as extinction), and they can be expected to have had an effect on the carbon-isotopic signature of sea water. It is possible that the $\delta^{13}\text{C}$ excursions reported here are connected with the other chemical events which took place around the Precambrian/Cambrian boundary and which were probably instrumental in the Cambrian metazoan radiation event.

If, as contended, the $\delta^{13}\text{C}$ excursions reported here are due to fluctuations in oceanic $\delta^{13}\text{C}$, they should be recorded in other boundary sections and could be a means of correlation, independent of the palaeontology, in a similar way to that proposed for magnetic polarity stratigraphy³¹. This could be very useful with some boundary profiles where, because of the facies developed, fossils are poorly represented.

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Planktonic foraminiferal ontogeny and new perspectives for micropalaeontology

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Planktonic foraminifera are some of the most widely used microfossils for dating marine sedimentary rocks and the calcareous test has been used to reconstruct their depositional history over the past 120 million years¹. By tracing the successive growth stages of extant representatives of this group, we have obtained criteria which identify pre-adult forms that, traditionally, have been disregarded in foraminiferal research. Thus, large quantities of "...unidentifiable miscellaneous juveniles ..." have become available to help decipher the fossil record of marine environments. Comparative studies of growth series at species and supraspecific levels allowed the identification of three major species groups, each showing three different developmental stages. Their recognition introduces new possibilities for establishing a natural classification of planktonic foraminifera, for disclosing their evolutionary history and for testing evolutionary models in general.

Despite the early recognition of the importance of reconstructing the sequence of events during planktonic foraminiferal growth²⁻⁸, only recently has the first comprehensive and comparative work appeared⁹. Departing from previous procedures, we followed an ontogenetic approach by considering the complete series of individual ontogenetic stages of each of the 10 species present in our samples. We used pre-adult tests collected from the living plankton offshore Barbados to minimize interference by additional carbonate layers secreted during later growth stages⁷, and to exclude post-lifetime alterations of the test¹.

Initially, the pre-adult morphology of a species had to be learned by backtracking the successive growth stages of trans-

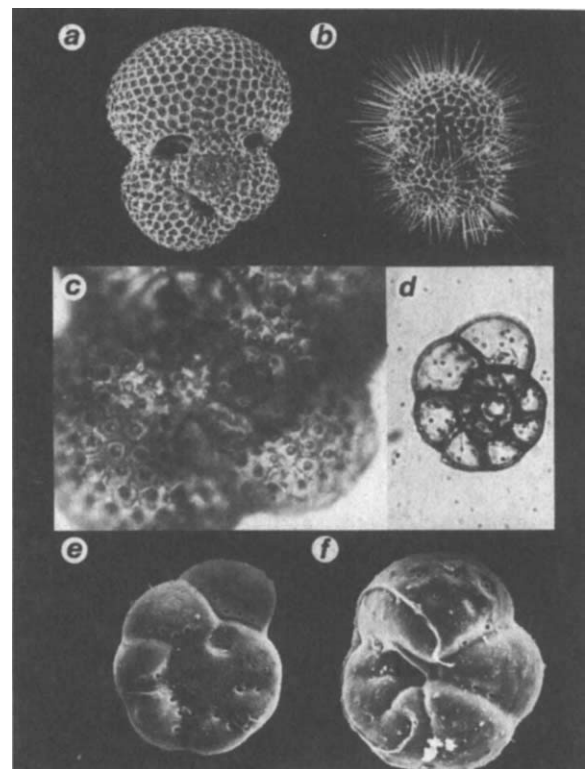


Fig. 1 Scanning electron (a, b, e, f) and transmission light (c, d) micrographs, illustrating the pre-adult morphology of *G. sacculifer* (Brady) as obtained from backtracking the successive growth stages of transparent adult specimens. a, Spiral view of adult specimen ($\times 88$). b, Umbilical view of adult specimen ($\times 79$). c, Same specimen as in a, showing the pre-adult growth stages contained in the adult test ($\times 194$). d, Spiral view of a typical juvenile ($\times 321$). e, Spiral view of a juvenile, compare with c and d ($\times 429$). f, Umbilical view of an early neanic form, showing the abrupt morphological transition from the juvenile stage (penultimate chamber) to the neanic stage (last chamber; $\times 352$).

parent adult specimens under the light microscope (Fig. 1c, d). This enabled us to determine the taxonomic identity of the individual pre-adult stages and to reverse the backtracking process in order to document morphological change during ontogeny.

Most species completed at least three phases of growth. During the initial (juvenile) stage, as well as the final (adult) stage, growth is morphologically conservative. Confined by rather spasmodic morphological changes, the intermediate (neanic) stage shows rapid transformation of virtually all features during its development.

Three species groups exhibiting characteristic ontogenetic morphology could be discerned (Fig. 2). Their distinction is essentially based on the same criteria used in traditional foraminiferal classification: gross shape of the test and constituent chambers, presence or absence of spines, position of the aperture(s) and texture of the test wall¹⁰. Remarkably the species groups distinguished here coincide with a subdivision in adult wall-textural types as conceived recently¹¹.

Group 1, comprising the species *Globigerinella aequilateralis* (Brady), *Globigerinoides conglobatus* (Brady), *Globigerinoides ruber* (d'Orbigny), *Globigerinoides sacculifer* (Brady) and *Orbulina universa* (d'Orbigny), shows striking morphological changes during ontogeny (Fig. 2a-d). Juveniles of this group have few large pores concentrated along sutures, a smooth test wall with sparse, sparsely distributed delicate spines and an extra-umbilical aperture. Then, when a size of $\sim 80 \mu\text{m}$ is reached, abrupt morphological changes occur, marking the onset

of the neanic stage. Well-defined, densely distributed spines appear on all chambers, pores spread over the entire chamber surface and the cancellate wall texture emerges (Fig. 1f). During the neanic phase, the number of chambers per whorl decreases and the main aperture shifts towards its ultimate position. Finally, at a size of $\sim 200\ \mu\text{m}$, advanced features such as secondary apertures herald the transition from the neanic to the adult stage of growth.

Group 2, which includes the species *Globorotalia menardii* (Parker, Jones and Brady) and *Neogloboquadrina dutertrei* (d'Orbigny), ontogenetically resembles group 1 (Fig. 2e-h). Consistent differences are the pustulate instead of spinose wall texture, the less-pronounced apertural shift during the neanic stage and a morphologically gradual neanic-adult transition. Additional characteristics of *G. menardii* comprise the development of a keel during the neanic stage and the absence of a cancellate wall texture.

Group 3, consisting of *Candeina nitida* (d'Orbigny), *Globigerinita glutinata* (Egger) and *Globigerinita juvenilis* (Bolli), retains most morphological features throughout ontogeny (Fig. 2i-l). Numerous minute pores may cover the non-spinose, pustulate test from as early as the second chamber. The apertural shift is a prominent feature of the neanic stage but the neanic-adult transition could be defined only in *C. nitida*, where chambers provided with numerous sutural apertures cover the umbilicus.

Planktonic foraminiferal classification, taxonomy and phylogeny has relied solely on adult morphological features⁹⁻¹². Our study of growth series of species reveals that major taxonomic characters change during the ontogeny of individual species. If the present, adult-based taxonomy were applied to the successive ontogenetic stages of, for instance *G. ruber*, it would have to be assigned to three different genera. As integral

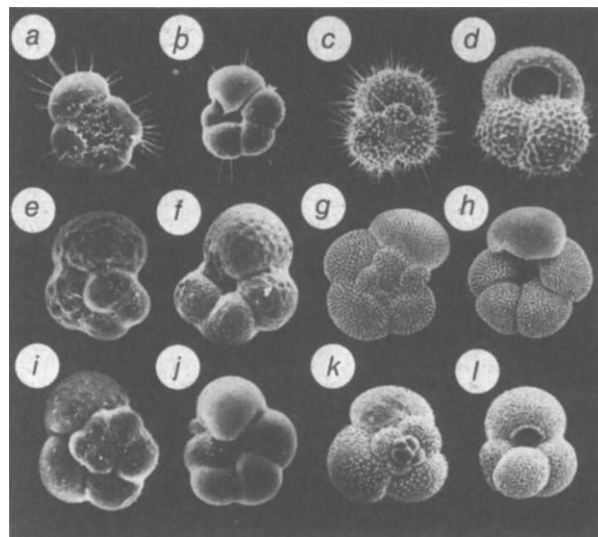


Fig. 2 Scanning electron micrographs of the adult versus pre-adult morphology of three species representing the three species groups distinguished by their ontogeny. *a-d*, *G. ruber* (group 1); *a, b*, spiral ($\times 449$) and umbilical ($\times 469$) view of juvenile, *c, d*, same views ($\times 120$, $\times 147$) of adult specimens. *e-h*, *N. dutertrei* (group 2); *e, f*, spiral ($\times 290$) and umbilical ($\times 304$) view of early neanic forms, *g, h*, same views ($\times 74$, $\times 62$) of adult specimens. *i-l*, *G. juvenilis* (group 3); *i, j*, spiral ($\times 653$) and umbilical ($\times 805$) view of early juvenile, *k, l*, same views ($\times 143$, $\times 136$) of adult specimens. Striking differences between ontogenetic phases are observed, involving taxonomically important characteristics such as wall texture, spine and pore distribution, shape and number of chambers in the last whorl, and position of the aperture.

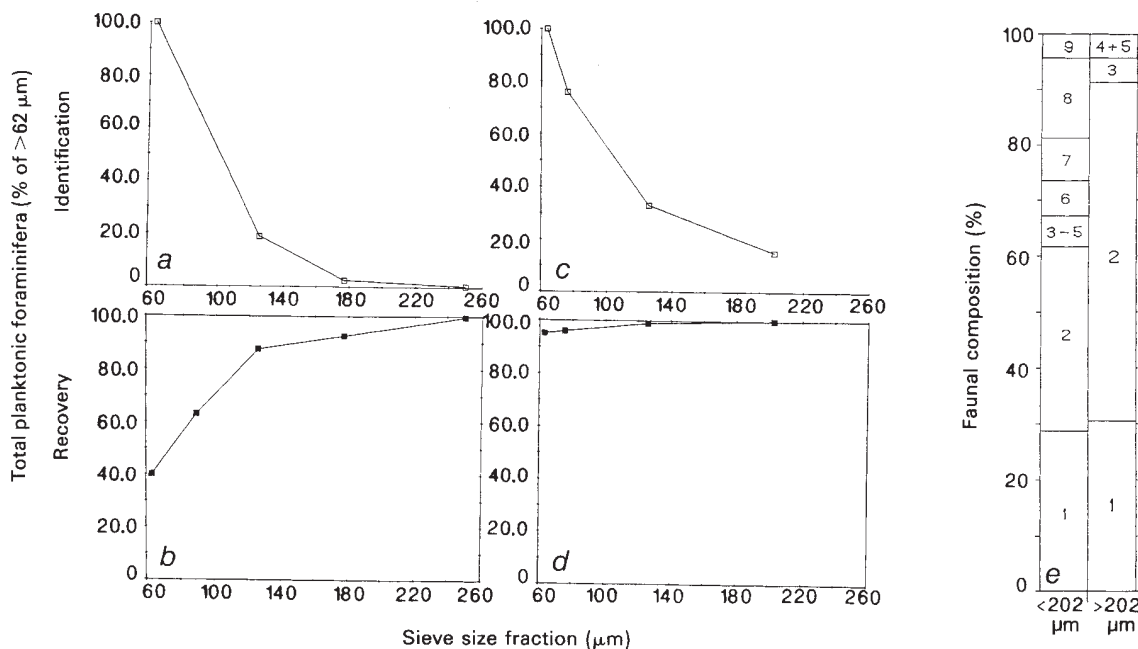


Fig. 3 Effect of the ontogenetic analytical approach on recovery, identification and species composition of planktonic foraminifera in relation to sieve mesh size. Sediment sample preparation methods traditionally use 125- μm or larger-sized sieve mesh fractions². Living plankton is usually collected by plankton nets of 202 μm or greater mesh size¹⁶. *a*, Recovery in sediment surface samples from the Santa Barbara Basin, Pacific Ocean, demonstrating the exponential growth of the recovery of specimens with decreasing mesh-size (after ref. 16). *b*, Single sediment surface sample from offshore Madagascar, Indian Ocean, showing rapidly diminishing identification frequencies towards the fine fractions when conventional identification criteria are used (modified after ref. 2). *c*, Surface water, 10- μm plankton net samples from offshore Barbados, Caribbean Sea. Recovery frequency runs parallel to that of sediment surface samples in *a*. *d*, Same sample as in *c*, using identification criteria based on ontogenetic morphology. The identification frequency remains very high in contrast to low values attained by using conventional (adult) morphological criteria (*b*). *e*, Changed species composition in the >202- μm compared with the <202- μm sieve fraction of a 10- μm plankton net sample (same as in *c, d*). 1, *G. ruber*; 2, *G. sacculifer*; 3, *N. dutertrei*; 4, *Globigerinita aequilateralis*; 5, *O. universa*; 6, *Globorotalia anfracta*; 7, *Globigerinita glutinata*; 8, *G. juvenilis*; 9, unidentified. Note the absence of *G. anfracta*, *G. glutinata* and *G. juvenilis* in the >202- μm fraction and the greatly reduced relative frequency of *G. sacculifer* in the <202- μm fraction.

parts of ontogeny, the morphology of the juvenile, neanic and adult phases together provide a new and comprehensive set of distinguishing features, evoking a reconsideration of our theory about planktonic foraminiferal species.

Although phylogeny has essentially been considered as a series of ontogenies^{13,14}, the comparison of adult morphological characteristics has traditionally been used to reconstruct planktonic foraminiferal evolutionary lineages^{9,12}. We expect that comparative ontogeny, rather than adult morphology alone, will produce a balanced and natural classification, taxonomy and phylogeny^{1,3} which, at present, suffers from adaptive adult morphological convergences and repetitive evolution^{1,12}. Using comparative ontogeny to infer evolutionary lineages reliably, we can more accurately define lineage-zones, regarded as "... one of the best assurances of reliable time correlation on a biostratigraphic basis"¹⁵.

Most importantly, ontogenetic morphology allows the identification of the pre-adult forms which constitute the bulk of a foraminiferal sample^{2,16}. The 125- μm , 149- μm or 202- μm sieves generally used to recover adult forms also induce the single most critical artefact in faunal analyses². With decreasing mesh size, recovery of specimens from a sample increases exponentially, identification frequencies decline correspondingly and marked shifts in faunal composition occur (Fig. 3). A pilot survey of a 10- μm plankton net sample from offshore Barbados shows an exponentially increasing specimen recovery, similar to that reported elsewhere^{2,16}. However, when ontogenetic morphology is used as a tool for identification, 95% of the small (62–125 μm) sieve-size fraction is still identifiable (Fig. 3d). Faunal analysis of the same sample shows marked changes in species composition relative to sieve mesh size (Fig. 3e). Large species dominating the >202- μm fraction have greatly diminished relative frequencies in the <202- μm fraction, whereas small species start to appear in considerable quantities.

Small species, accounting for about one-third of all extant taxa¹, have a largely unexplored potential due to their hidden occurrence among unidentified pre-adult forms. Together, their abundance and great diversity may give a detailed biostratigraphical analysis of fossil, adult-poor, shallow marine sediments. Faunal composition, as recognized in the small-sized fraction, will lead to more accurate and stable reconstructions of late Tertiary palaeoceanography and palaeoclimatology¹⁶, which rely mainly on patterns of foraminiferal distribution¹.

Planktonic foraminiferal ontogeny yields a new and penetrating micropalaeontological approach which will contribute towards a better understanding of planktonic foraminifers as a group and their application in biostratigraphy, palaeoceanography and palaeoclimatology.

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Acoustic cavitation generated by microsecond pulses of ultrasound

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Under extreme acoustic stress, liquids can be caused to rupture, with the transient generation of a vapour cavity. The subsequent collapse of these cavities is normally violent enough to be observed with the unaided eye or ear, and often generates free radicals due to the high temperatures and pressures associated with the collapse. Flynn¹ has calculated that microsecond pulses of ultrasound with peak intensities in the range 10–30 W cm⁻² can generate transient cavitation in water. Because some diagnostic ultrasound systems^{2,3} now in clinical use generate microsecond length pulses with temporal peak intensities >100 W cm⁻², there is reason to believe that this mechanism could operate in diagnostic conditions in aqueous media. We show here that ultrasonic pulses as short as one cycle at a frequency of 1.0 MHz give rise to luminescence flashes characteristic of violent cavitation. This provides the first experimental confirmation of Flynn's theoretical calculations.

Because the pulses produced by diagnostic ultrasound generators are rich in harmonics and short in time, we decided not to utilize the commonly used cavitation detection criterion—the acoustic detection of a shock wave or subharmonic frequency components—as there is little evidence that cavitation collapses in this short pulse regime can be detected acoustically. Rather, we have used a photometric detection system based on the observation, through a photomultiplier tube, of light produced by the cavitation bubble. This light emission results from the oxidation of luminol by the free radicals generated during the collapse of a cavity and should properly be termed chemiluminescence.

An essential ingredient of the cavitation generation system is that the acoustic pressure amplitude of the sound field is increased linearly with time, at a reproducible rate, until cavitation occurs. The experimental system will be described in more detail in a later report, a similar apparatus is described elsewhere⁴. The heart of the system is a ramp generator that drives both a strip recorder and the amplitude modulation input of a function generator. The d.c. voltage level on the strip chart record is linearly related to the peak-to-peak output of the function generator. This signal is amplified and delivered to a focused transducer (resonance frequency 1.0 MHz) that radiates into an echo-free chamber (wall lined with acoustic absorber) filled with distilled water saturated with argon, and containing luminol. The chart record is calibrated in units of megapascals by inserting a small, calibrated hydrophone (Medicoteknik Institut, PVDF ultrasonic probe, outer diameter, 1.0 mm) into the focal region of the sound field during simulated measurements at low amplitudes.

Light generated by a cavity collapsing in the focal region is detected by a photomultiplier tube and the resulting pulse is preamplified and fed into a pulse height discriminator. The

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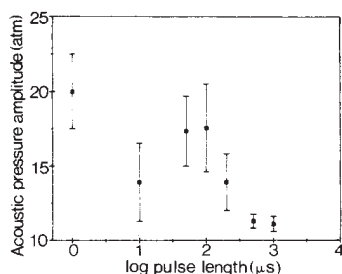


Fig. 1 Variation of the acoustic cavitation threshold of water containing luminol with pulse length for a duty cycle of 1:10. The points are the average of at least 10 independent measurements and the error bars represent $\pm$ one standard deviation. The carrier frequency was 1.0 MHz and the liquid was saturated with argon. The abscissa plots the logarithm (to the base 10) of the pulse length to compress the scale. Thus, an abscissa value of 3 implies that about 1,000 cycles were contained in the pulse, 2 means 100, 1 means 10, and 0 indicates that the pulse contained essentially one cycle.

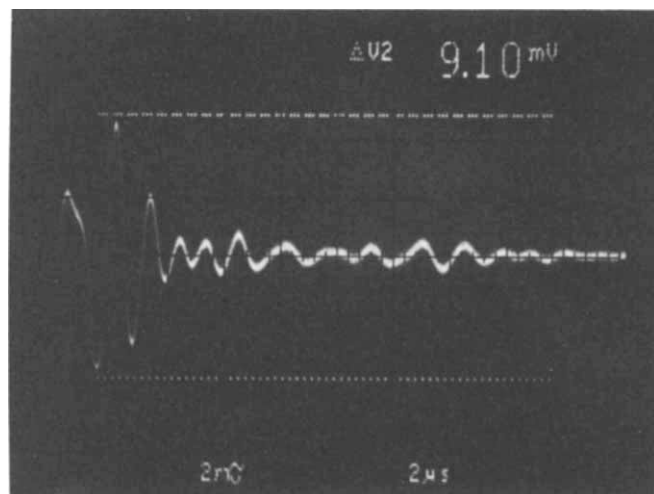


Fig. 2 An oscilloscope photograph of the temporal variation of the acoustic pressure near the focus of the sound field as measured by a calibrated miniature hydrophone. This pulse is intended to mimic those used in diagnostic ultrasound scanners.

discriminator level determines the sensitivity of the apparatus and is set to 600 mV, which is well above the average electrical background level (~ 150 mV) but below the typical chemiluminescence pulse height (~ 1.0 V). After discrimination, the signal passes through a noise gating network that either ignores the signal (no decision) or resets the system (cavitation detected). The noise gating network is a type of coincidence counter that requires that two pulses be observed within a 100-ms time interval. The reason for this criterion is that we have discovered through experience that a cavitation 'event' typically generates several optical flashes during 100 ms while the background noise is randomly distributed in time. The noise gating thus significantly lowers the false count rate. The system is reset by an immediate reduction of the acoustic pressure amplitude to zero for a quiescent period of 300 s; after this resting period is over, the acoustic pressure is again ramped until a cavitation event occurs and the sequence is repeated.

We have taken measurements of the cavitation threshold as a function of pulse length and of the length of time between pulses (duty cycle). A duty cycle of 1:10 means that the sound field is on for 1 time unit and off for the succeeding 9 time units. (This is a much longer duty cycle than used clinically by one to two orders of magnitude.)

Figure 1 shows the measured values of the threshold acoustic pressure amplitude required to initiate cavitation in distilled water containing luminol, as a function of pulse length, with a duty cycle of 1:10. Similar behaviour is observed for duty cycles of 1:3, 1:5 and 1:20. It is the temporal peak acoustic pressure rather than the average intensity that is important in the generation of cavitation effects⁵. An approximate value of the temporal peak intensity can be obtained by assuming a plane wave and using the relation $I = P_a^2 / 2\rho c$, where P_a is the temporal peak acoustic pressure, ρ is the density of the liquid and c the velocity of sound in the liquid.

Each data point presented is the average of at least 10 individual measurements. For the critical data point at an abscissa value of 0 (corresponding to pulses generated by a diagnostic ultrasound generator), we have taken more than 30 separate measurements on three different days with three different liquid samples. On one occasion, a second operator made the measurements (although the system is essentially automated). We thus have confidence in the validity of this measurement. Furthermore, at a duty cycle of 1:10, and at values of the temporal peak acoustic pressure only slightly higher than that determined as the threshold, single cycle pulses of 1-MHz ultrasound give light emission that is easily visible to the naked eye.

Figure 2 shows a photograph of the temporal variation of the acoustic pressure as measured by our miniature probe hydro-

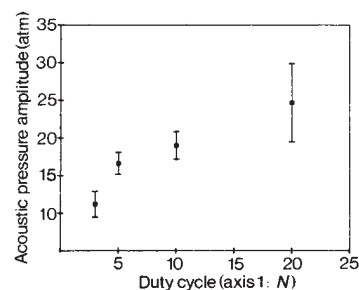


Fig. 3 Variation of the acoustic cavitation threshold of water containing luminol with duty cycle for a pulse length of $\sim 1\mu$ s. The carrier frequency was 1.0 MHz and the liquid was saturated with argon.

phone. Note that there is some ringing of the transducer but only at a much reduced amplitude. This pulse closely resembles the scanning mode pulse of a diagnostic ultrasound system which typically has a large negative cycle followed by a large positive cycle. Furthermore, this pulse is also similar to that used by Flynn¹ in his theoretical studies.

We have also examined the dependence of the cavitation threshold on duty cycle for the 'one cycle' pulse. These data are shown in Fig. 3. We have been concerned that the cavitation nuclei may respond to the pulse repetition frequency rather than the individual pulses. However, since the acoustic pulse used here dies out within a few microseconds, (well before the beginning of the next pulse), there is good reason to believe that these pulses act independently of each other. The data in Fig. 3 essentially confirm this fact showing an asymptotic behaviour at larger duty cycles. Furthermore, Marston and Unger⁶ have presented evidence that single pulses of negative pressure can produce cavitation in water.

Note that we have established an arbitrary definition of the cavitation threshold as that value of the peak temporal acoustic pressure at which the output pulses reaching the pulse height discriminator from the photomultiplier tube have values of 600 mV or larger. This requirement is a working definition in that this discriminator setting permits us to obtain a desirable signal-to-noise ratio. It is quite conceivable that a more sensitive detection system would indicate lower values of the threshold. Our definition of cavitation involves the production of light by the collapsing cavity. Sonoluminescence is a reasonably well

understood phenomenon^{7,8} that is apparently the result of the radiative recombination of free radicals produced by the collapsing cavity⁹. However, the light emission that we observe is probably not sonoluminescence from within the cavity itself (produced by the recombination of free radicals); rather, it is light from the bulk of the liquid resulting from free radicals which 'escape' from the cavity and react with the luminol. Luminol has been used by several researchers (see, for example, ref. 10) to increase the output of light emissions from acoustic cavitation and the specific oxidation reactions that lead to light emission have been studied in some detail^{11,12}. What is most important, and relevant to this work, is that the presence of free radicals are required for light emission to occur. Because these free radicals are highly reactive, and pose a threat to biological tissues, it is their presence that is important, not the light emission *per se*.

The light emission from the insonified area we observed can be seen by the naked eye, and appears in the form of 'streaks' rather than 'flashes'. We interpret this as being due to the relatively long lifetime of the oxidation reaction (as long as 50 ms according to Finch¹⁰ and the rapid movement of collapsing cavities through the focal region due to radiation pressure forces. However, we have recently observed intense light emission from a form of 'stable' cavitation, in which bubbles pulsate violently for many cycles, with the use of an image intensifier tube as a light detector¹³. Because the temporal peak acoustic pressures are quite large, we should not expect that individual cavitation bubbles would survive the collapse phase and remain intact. It is possible, however, that the collapse and bubble breakup would effectively 'seed' the liquid with several new microbubbles that could act as cavitation nuclei¹⁴. The nearly continuous light emission suggests this form of 'steady' cavitation.

Finally, we draw attention to the fact that biological systems possess stabilized gas nuclei that can be activated by reduced pressures, either statically, as in the case of decompression sickness^{15,16}, or dynamically, as in the case of the biological effects of ultrasound¹⁷⁻²¹. These nuclei seem to exist in appreciable numbers in a variety of biological tissues, and our new evidence gives reason to believe that single cycle pulses of ultrasound could activate these nuclei in a violent manner. Accordingly, the data presented here may have important implications for clinical use of such ultrasound systems and imply that extensive risk assessment studies ought to be carried out.

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Potent ulcerogenic actions of platelet-activating factor on the stomach

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Platelet-activating factor (PAF) is an endogenous phospholipid which has been implicated as a mediator of allergic and inflammatory processes¹. It is synthesized and released by neutrophils, platelets, macrophages, monocytes, basophils and endothelial cells²⁻⁸, and is a potent platelet-aggregating agent, a vasodilator, increases vascular permeability, stimulates neutrophil aggregation and degranulation⁹ and induces release of lysosomal enzymes¹⁰. A role for PAF in the hypotension associated with endotoxin shock^{11,12} and in necrotizing enterocolitis¹³ has recently been suggested. As there is an association between septic shock and acute gastric damage¹⁴, we propose that PAF is an endogenous mediator of ulceration in the stomach. Indeed, as reported here, intravenous (i.v.) infusion of PAF to rats, at doses of 20–200 pmol per kg per min, resulted in the formation of extensive haemorrhagic erosions in the gastric mucosa. The ulcerogenic actions of PAF are not attributable solely to its hypotensive actions and were not mediated via effects on platelets or cyclooxygenase products, nor via histamine H₁, H₂ or α -adrenergic receptors. PAF is the most potent gastric ulcerogen yet described and its endogenous release may underlie or contribute to certain forms of gastric ulceration.

PAF (1-*O*-alkyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine; Sigma) was infused i.v. (100 ng per kg per min) to pentobarbitone-anaesthetized male Wistar rats for periods of 5–20 min. The stomachs were removed immediately after the PAF infusion or at 30–180 min after the infusion. With this dose, macroscopically visible gastric erosions were present in all of the treatment groups. As the most consistent level of damage was produced in the group infused with PAF for 20 min with the stomach removed 60 min later, this protocol was used in all subsequent experiments. To test the relationship between dose of PAF and the extent of gastric damage, PAF was infused i.v. at doses of 10–100 ng per kg per min (20–200 pmol per kg per min); 1 h later the stomachs were removed, opened and the mucosa was photographed using colour transparency film. Samples from the fundic region were fixed in neutral-buffered formalin for subsequent histological analysis. The severity of macroscopically visible damage was scored, independently, by two observers in a randomized, blind manner and assigned a score on a scale from 0 (normal) to 3 (severe) (see Fig. 1 legend).

All doses of PAF produced areas of macroscopically visible gastric hyperaemia, with doses of 30–100 ng per kg per min producing extensive, severe hyperaemia and haemorrhage (Fig. 1). As evaluated histologically, the damage caused by these doses of PAF was characterized by extensive vascular congestion, extravasation of erythrocytes, destruction of the surface epithelium, dilatation of glands and focal regions of necrosis extending throughout the depth of the mucosa (Fig. 2). The incidence of these effects increased with an increase of the dose of PAF infused. In addition to congestion of mucosal and submucosal blood vessels with erythrocytes, aggregates of polymorphonuclear leukocytes were frequently observed, particularly at the base of the mucosal glands.

As has been reported previously⁹, infusion of PAF had significant dose-related hypotensive effects. At doses of 10, 30, 50 and 100 ng per kg per min, PAF produced decreases in mean systemic arterial blood pressure of 18 ± 4 , 48 ± 4 , 62 ± 4 and 64 ± 9 mm Hg, respectively ($n = 5$ per group). Although it is well established that both clinical and experimental shock are associated with acute gastric ulceration¹⁵, a reduction in blood pressure

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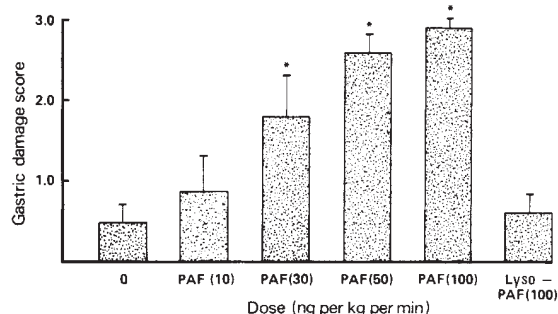


Fig. 1 Gastric damage scores for rats given PAF, lyso-PAF or vehicle alone i.v. for 20 min. Damage was assessed from colour transparencies taken 1 h after completion of the infusion. A score of 0 was assigned if the mucosa appeared normal. A stomach exhibiting haemorrhage or erythema was assigned a score of 1 (diffuse), 2 (moderate, multiple sites) or 3 (severe, extensive). The scoring was done independently by two observers in a randomized, blind manner and there was a highly significant correlation ($r = 0.87$; $P < 0.001$) between the scores assigned by the two observers. Asterisks indicate groups which differ significantly ($P < 0.05$) from the control group.

Table 1 Effects of hypotensive agents on gastric mucosal damage

Compound	Dose (μg per kg per min)	Damage score	% With haemorrhage	Δ BP (mm Hg)
Vehicle	—	0.5 ± 0.2	0	1 ± 2
PAF	0.05	$2.6 \pm 0.2^*$	75	$62 \pm 4^*$
Prostacyclin	0.5	0.6 ± 0.2	0	$55 \pm 4^*$
Isoprenaline	0.5	0.3 ± 0.2	0	$50 \pm 3^*$
Nitroprusside	10.0	$1.4 \pm 0.4^\dagger$	0	$72 \pm 2^*$

Hypotensive agents were administered i.v. for 20 min; 1 h after completion of the infusion, the stomach was removed and the damage assessed as described in Fig. 1 legend. The presence of haemorrhage was determined histologically and is expressed here as the per cent of the total number of rats in which haemorrhage was observed. In the control group, vehicle (0.25% bovine serum albumin in normal saline) alone was infused. Isoprenaline hydrochloride (Sigma) and sodium nitroprusside (BDH) were dissolved in normal saline. Prostacyclin (Wellcome Foundation Ltd) was dissolved initially in 1 M Tris buffer (pH 8.5) then diluted in 1.25% sodium bicarbonate. Results are the mean $\pm$ s.e.m. for 5–8 rats per group. Δ BP represents the decrease in mean arterial blood pressure during the infusion of the compound. With all agents, the peak fall in blood pressure was observed within 1 min of the start of the infusion and the depression of blood pressure was maintained throughout the infusion period. After termination of the infusion, the blood pressure returned to control levels within 10 min.

* $P < 0.01$; $^\dagger P < 0.05$, significant difference compared with the control group.

by pharmacological vasodilators is not generally associated with such mucosal damage. However, to determine whether the fall in blood pressure produced by PAF was itself sufficient to induce gastric damage in the present study, some other hypotensive agents were administered i.v. according to the same protocol. Isoprenaline (0.5 μg per kg per min), prostacyclin (0.5 μg per kg per min) and nitroprusside (10 μg per kg per min) each produced a rapid fall in blood pressure of 50–70 mm Hg which was comparable to that induced by high doses of PAF and was maintained throughout the period of infusion (Table 1). Only infusion of nitroprusside produced macroscopic gastric damage, but this was significantly less severe ($P < 0.05$) than that produced by a dose of PAF (30 ng per kg per min) which elicited a significantly smaller ($P < 0.05$) fall in blood pressure. Furthermore, histological evaluation revealed that none of these hypotensive agents produced haemorrhagic erosions in the gastric mucosa. Thus, the fall in blood pressure induced by PAF

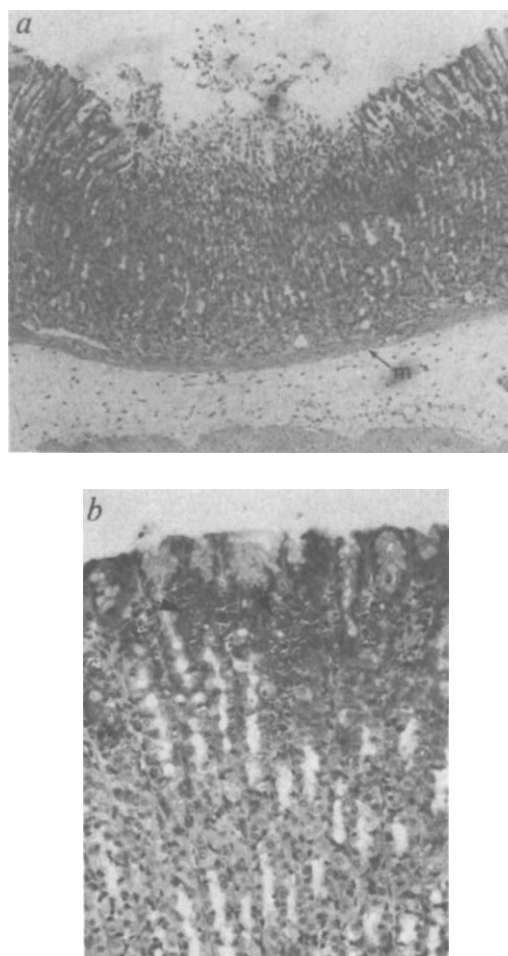


Fig. 2 *a*, Haemorrhagic erosion in the gastric mucosa of a rat given PAF (100 ng per kg per min) for 20 min. The tissue was fixed 1 h after completion of the infusion. Note that the necrotic region extends down to half the depth of the mucosa and does not penetrate the muscularis mucosae (m). Extruded cells (e) can be seen in the lumen. (Haematoxylin and eosin stain. $\times 39$.) *b*, Mucosal vasocongestion (arrows) in gastric mucosa of rat given PAF as for *a*. This engorgement extended throughout the entire depth of the mucosa. Macroscopically, this region appeared hyperaemic. (Haematoxylin and eosin stain. $\times 96$.)

would be insufficient alone to produce the observed mucosal damage.

We investigated whether the effects of PAF are shared by its structurally similar precursor and breakdown product, lyso-PAF (1-*O*-alkyl-*sn*-glyceryl-3-phosphorylcholine). Lyso-PAF (100 ng per kg per min) given i.v. had no significant effect on the gastric mucosa, as assessed either macroscopically (mean score of 0.6 ± 0.2) or histologically, nor did it affect blood pressure. A higher dose of lyso-PAF (10 μg per kg per min), which also failed to alter the blood pressure, produced diffuse mucosal hyperaemia (mean score of 1.4 ± 0.3). The level of damage was less than that produced by PAF at 30 ng per kg per min, indicating a difference in ulcerogenic potency of over 3,000-fold, while a dissociation between the gastric-damaging actions and any hypotensive actions of lyso-PAF was clearly demonstrated.

Although PAF has been shown to be a vasodilator in most organs and to increase vascular permeability, its actions on the gastric vasculature are not yet known. Histamine, a vasodilator, has been suggested to have pro-ulcerogenic actions¹⁶, perhaps through its ability to increase capillary permeability. Several agents which have vasoconstrictor actions in the gastric mucosa

have also been shown to be pro-ulcerogenic: for example, thromboxane A₂ (ref. 17) and noradrenaline¹⁸. Release of these endogenous mediators could, therefore, be involved in the ulcerogenic actions of PAF. To test for any such role, an inhibitor of cyclooxygenase (indomethacin), an α -adrenergic receptor antagonist (phentolamine) or a histamine H₁-receptor antagonist (mepyramine) was administered before i.v. infusion of PAF (50 ng per kg per min) at a dose yielding a damage score of 2.6 ± 0.2 . Indomethacin (5 mg per kg subcutaneous) administered 45 min before PAF caused a significant ($P < 0.001$) increase in the damage score (3.0 ± 0 ; $n = 4$). Pretreatment with phentolamine (10 mg per kg i.v.) or mepyramine (5 mg per kg i.p.) did not significantly affect the extent of damage induced by PAF (mean score 2.0 ± 0.2 , $n = 5$ and 2.0 ± 0 , $n = 3$, respectively). The ulcerogenic actions of PAF therefore appear to be independent of the release of these mediators.

Although resting gastric acid secretion in the anaesthetized rat is low, intragastric instillation of 0.1 M HCl (3 ml) before PAF infusion (50 ng per kg per min) did not significantly augment the severity of macroscopic damage, although the regions of mucosal haemorrhage were stained black. Furthermore, i.v. administration of antisecretory doses of the H₂-receptor antagonist cimetidine (50 mg per kg) 15 min before infusion of PAF (100 ng per kg per min) did not significantly reduce the severity of the gastric damage (mean score of 2.8 ± 0.3 ; $n = 4$). Thus, unlike the case with many experimental models of gastric damage, the presence of a high intraluminal concentration of acid is not a prerequisite for PAF-induced ulceration.

Despite its name, PAF does not cause platelet aggregation in the rat, nor does it cause the thrombocytopenia that is associated with infusion of PAF in other species⁹. However, it might stimulate the release of biologically active mediators from platelets which could contribute to the ulcerogenesis. This possibility was studied in rats made thrombocytopenic 24 h previously by i.v. injection of an anti-platelet serum raised in rabbits against rat washed platelets¹⁹. Reduction of the number of circulating platelets by 85–90% below that of control rats treated with normal rabbit serum did not reduce the damage (mean score 2.2 ± 0.6 ; $n = 3$) produced by PAF (50 ng per kg per min).

Our studies demonstrate that PAF is a potent ulcerogenic agent in the rat stomach. In a previous study on ischaemic bowel necrosis, haemorrhagic lesions were observed in the rat gastric mucosa after i.v. injections of very high doses of PAF in combination with endotoxin¹³. While the hypotensive actions of PAF could contribute to its ulcerogenic actions, especially at the higher doses, this is unlikely to be the sole mechanism. However, a local vascular action of PAF may well be important. Indeed, the histological observations of vascular congestion suggest that stasis may be a precipitating event in the ulceration process. Stasis could occur as a result of vasoconstriction, exudation of plasma, or through aggregation of one or more of the cellular components of blood. PAF has potent pro-aggregatory and degranulatory effects on neutrophils both *in vivo*²⁰ and *in vitro*²¹. Infusion of PAF to rabbits leads to substantial neutropenia, perhaps as a result of sequestration of neutrophil aggregates in the lung and other tissues^{20,22}. Sequestration of neutrophil aggregates in the stomach, as observed in the histological sections taken after PAF administration, could thus contribute to congestion of the mucosal vasculature. Generation of free radicals and release of lysosomal enzymes following neutrophil aggregation could also contribute to the tissue damage induced by PAF.

Gastric damage was observed with a dose of PAF as low as 20 pmol per kg per min, the total dose administered over 20 min being 400 pmol per kg. Concentrations of PAF of 4.7 nM have been found in the blood of rats during endotoxin shock; this concentration could be achieved by an infusion of PAF at 300 pmol per kg¹². Hence, the doses of PAF which produced extensive gastric damage in the present model are within the range of concentrations found *in vivo* in a model of a disease associated with acute gastric ulceration. The use of specific PAF

antagonists will help to clarify the contribution of endogenous PAF to such gastric damage. Whether the release of PAF from circulating or entrapped blood cells under other circumstances, such as after haemorrhagic shock, also contributes to gastric damage is not yet clear. It is conceivable that in conditions characterized by infiltration of inflammatory cells, or in the sequence of events following local tissue damage, PAF may be released directly into the mucosa. Thus, both systemic and local release of PAF could initiate or further amplify gastric ulceration under pathological conditions.

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1-Methyl-4-phenylpyridine is neurotoxic to the nigrostriatal dopamine pathway

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Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is neurotoxic to cerebral dopaminergic neurones in several animal species, and can cause parkinsonism in man^{1–5}. The mechanism of this action may be indirect. MPTP is oxidized in the brain to a pyridinium species, 1-methyl-4-phenylpyridine (MPP⁺)⁶. This oxidation is greatly decreased by inhibition of monoamine oxidase B⁶, as are the biochemical effects of MPTP in the mouse⁷ and its neurotoxicity in the monkey⁸. We now show that MPP⁺ exerts a powerful neurotoxic action on the nigrostriatal dopamine system of the rodent.

The neurotoxic potential of MPP⁺ in the mouse was initially investigated by injecting MPP⁺ (10 µg) dissolved in distilled water into the ventricular system, a route selected to avoid the difficulties of penetrating the blood–brain barrier. Mice received one injection daily for 3 days, via chronically in-dwelling guide

Table 1 Neurotoxic action of MPP⁺ and MPTP infused into rat substantia nigra revealed as depletions of dopamine/metabolites in posterior striatum

Treatment	Dopamine (ng per mg tissue)	DOPAC	HVA (pg per mg tissue)	Serotonin	5-HIAA
Vehicle	2.70 ± 0.4	242 ± 23	166 ± 36	338 ± 62	281 ± 24
MPTP	2.44 ± 0.2	281 ± 17	144 ± 78	485 ± 41	307 ± 22
MPP ⁺	0.74 ± 0.1*	87 ± 9*	44 ± 8†	414 ± 44	274 ± 15

Male Sprague-Dawley rats (300–325 g) were implanted with chronically in-dwelling guide cannulae aimed at the substantia nigra zona compacta (Ant.2.2, Vert.1.8, Lat. ± 2.0)^{11,12} and were subsequently implanted subcutaneously (in the interscapular region) with two Alzet osmotic minipumps. MPTP (Research Biochemicals Inc.) or MPP⁺ dissolved in distilled water was continuously infused bilaterally via injection units connected to the pumps at a rate of 0.48 µl h⁻¹, containing 10 µg per day, for 4 days. Levels of dopamine, serotonin and their metabolites in the posterior striatum were determined by high-pressure liquid chromatography. Values are expressed as the mean ± 1 s.e.m.; *n* = 5.

* *P* < 0.01 and † *P* < 0.05 compared with vehicle treatment; Mann-Whitney *U*-test.

cannulae, and were killed on day 4. At this time, striatal dopamine levels were reduced by 35%, and 3,4-dihydroxyphenylacetic acid (DOPAC) levels by 27%, with no reductions in levels of homovanillic acid (HVA), serotonin or 5-hydroxyindoleacetic acid (5-HIAA). Thus, MPP⁺ was shown to have actions on dopaminergic neurones, the modest reductions perhaps reflecting the difficulties encountered by MPP⁺ in penetrating the neostriatal complex from the ventricular system.

Consequently, in a second series of experiments, MPTP and MPP⁺ were infused directly into the dopaminergic cell body group in the substantia nigra of the rat. The rat was a particularly suitable species for these studies because it is relatively insensitive to the neurotoxic actions of MPTP^{6,9}. To minimize problems of nonspecific damage, MPTP and MPP⁺ (10 µg per 24 h) were infused bilaterally into the substantia nigra via chronically in-dwelling guides and injection units. Motor functions, including locomotor response, ability to climb and grip on a vertical wire grid, and the presence of rigidity in the limbs and trunk were recorded daily during 4 days of infusion. Rats were killed 1 day and 1 month after the infusions and the striatum was dissected for analysis of the content of dopamine and serotonin, and their metabolites, and for estimates of ³H-dopamine uptake into striatal synaptosomes.

Even when MPTP was infused continuously into the substantia nigra, there was no loss of striatal dopamine or its metabolites (Table 1). There was a small decrease in ³H-dopamine uptake into striatal synaptosomes 1 day after cessation of MPTP administration (vehicle, 0.91 ± 0.01; MPTP, 0.62 ± 0.15 pmol ³H-dopamine per mg protein per 6 min, *P* < 0.05, *n* = 4), but this was not present 1 month after MPTP treatment (vehicle, 1.05 ± 0.17; MPTP, 0.82 ± 0.08 pmol ³H-dopamine per mg protein per 6 min, *P* > 0.05, *n* = 4).

In contrast, MPP⁺ infused into the rat substantia nigra was markedly neurotoxic to dopamine function; striatal dopamine levels 1 day after the infusion were reduced by 73%, DOPAC levels by 64% and HVA by 27%, with no change in levels of serotonin or 5-HIAA (Table 1). Infusion of MPP⁺ caused a large decrease in ³H-dopamine uptake at both 1 day and 1 month post-infusion (1 day, 0.27 ± 0.04; 1 month, 0.36 ± 0.04 pmol ³H-dopamine per mg protein, both *P* < 0.001 compared with vehicle controls (results above), *n* = 4).

MPP⁺ infused into the substantia nigra caused profound motor deficits, in contrast to the less severe effects of MPTP. Both neurotoxins caused reductions in locomotion and loss of ability to coordinate movements of the front limbs, seen as an inability to grip on a vertical wire grid and so climb on the grid. However, the effects of MPP⁺ were more severe, with additional loss of the ability to use the hindlimbs to grip and climb on the vertical grid, and the development of rigidity in forelimbs, hindlimbs and trunk (Table 2). These effects developed within 12 h of the start of MPP⁺ infusion into the substantia nigra and were maximal by day 2. The overall impression of the effects of MPP⁺ treatment was the appearance of a severe motor impairment.

Table 2 Motor impairment caused by the bilateral infusion of MPTP and MPP⁺ into rat substantia nigra

Treatment	Locomotor activity	Grid test	Rigidity
Vehicle	92 ± 10.0	20 ± 0.0	0.0 ± 0.0
MPTP	40 ± 5.1*	9.3 ± 0.8*	0.0 ± 0.0
MPP ⁺	13 ± 1.4*	0.0 ± 0.0*	15.0 ± 0.0*

Locomotor activity was measured daily in individual photocell cages; interruptions of beams were recorded every 5 min and summed to give counts per 60 min. Locomotor depression was maximal by day 2; data are given for day 3 (± 1 s.e.m.). Deficits in limb movement were assessed using a vertical wire grid and scored as follows: (1) climbing upwards or sideways on grid; (2) maintaining position on grid for 30 s; (3) gripping grid with forepaws; (4) gripping grid with hind paws. Results are expressed as the total score (± 1 s.e.m.) for five test sessions on days 2–4 of infusion. Rigidity was noted as being present or absent in the forelimbs, the hindlimbs and the trunk, giving a maximum score of 3 for each animal. Results are expressed as the total score (± 1 s.e.m.) determined during five test sessions on days 2–4 of infusion. *n* = 5.

* *P* < 0.001 compared with vehicle treatment; one-way ANOVA followed by Dunnett's test.

These studies in the rodent emphasize that the oxidative metabolism of MPTP is required for expression of its neurotoxic action, and that one metabolite, MPP⁺, found in monkey and mouse brain after MPTP administration⁶, has powerful neurotoxic action on the nigrostriatal dopaminergic system. The previous uncertainty as to whether MPTP neurotoxicity results from the formation of MPP⁺ or from the oxidative processes forming MPP⁺ is thus partly resolved; MPP⁺ itself appears neurotoxic, although histological assessment of the extent and specificity of the neuronal damage caused by MPP⁺ is required. It remains possible that the oxidative processes forming MPP⁺ also have neurotoxic potential. However, the nature of the motor deficits and severity of the loss of striatal dopaminergic function caused by MPP⁺ may provide clues to the pathogenesis of Parkinson's disease.

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A large anion-selective channel has seven conductance levels

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Ion channels have generally been found to have two predominant conductance levels thought to be associated with 'open' and 'closed' states, but intermediate (subconductance) states have also been reported¹⁻³. We have now found that a large conductance, anion-selective channel in pulmonary alveolar epithelial cells⁴ can adopt any of six open levels of conductance that are integer multiples of 60–70 pS. The channel is usually either fully open or fully closed. The frequencies of the different conductance levels are inconsistent with the notion that there are six independent channels. We suggest that the channel consists of six conducting pathways in parallel, 'co-channels', with a shared gating mechanism that can synchronously render all of them non-conducting. Other channels with lower maximum conductance may operate in a similar way but multiple conductance levels would not easily be detected because of a less favourable signal-to-noise ratio.

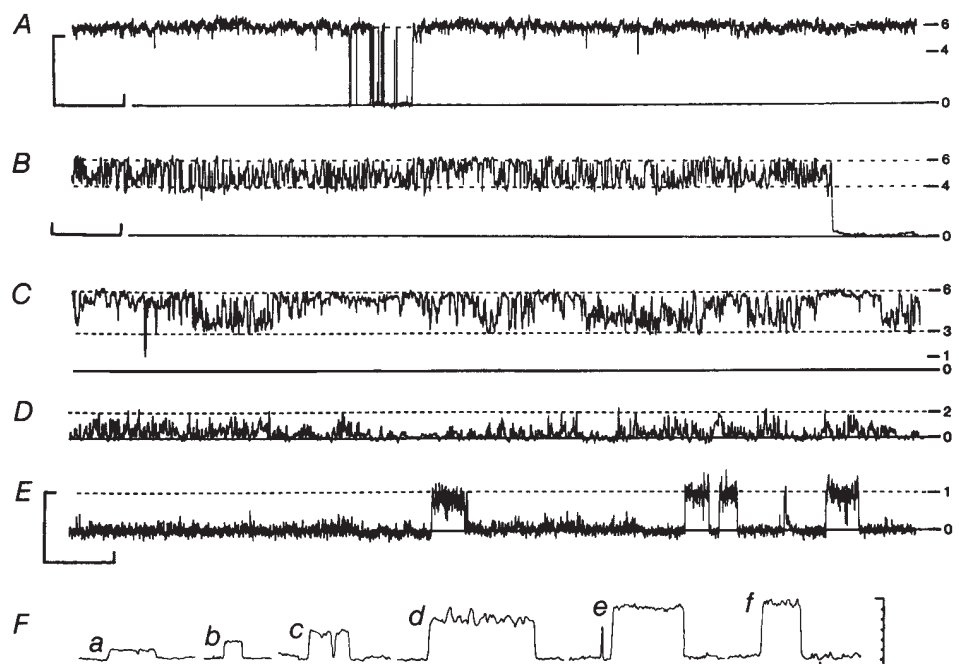
Currents were recorded at room temperature (21–25 °C) from inside-out patches excised from the apical surface of cultured mouse alveolar type II cells. Recording procedures are described elsewhere⁴. Excised patches were held at 0 mV and then stepped to positive or negative potentials to record currents. Following a step in potential, patches are seen to contain from 0 to 4 channels, each with a conductance of ~400 pS. In most patches, channels become inactivated with time, becoming completely inactivated within 6 s to 10 min and inactivating more rapidly the further the potential is stepped away from 0 mV. Inactivation could be reversed by returning to 0 mV. Thus, the large conduct-

ance anion channel in pulmonary epithelial cells has characteristics in common with large conductance anion channels described in other tissues⁵⁻⁷.

Selected current records, obtained from an excised patch in which there appears to be only one 400-pS channel following a voltage step from 0 to 57 mV, are shown in Fig. 1. This channel displays rapid inactivation so that no full openings are observed after 10 s. The records shown in Fig. 1 were chosen to illustrate the different current levels and types of channel behaviour. In Fig. 1A, the channel is open most of the time, closing several times near the middle of the record. The mean current through the open channel (upper level) is 23 pA, corresponding to a conductance of 404 pS (holding potential 57 mV, null potential 0 mV), similar to previous reports⁴ of the conductance for this channel. The noise level (flickering) is greater when the channel is open than when it is closed. This phenomenon is even more noticeable in Fig. 1B, C; B shows the current fluctuating between 21 and 15 pA, the latter being about two-thirds of the open current, whereas C shows the current fluctuating between 23 and 12 pA, the lower level being about half the maximum current. Occasionally, the current fluctuates between 0 and 7.5 pA (Fig. 1D) and, less frequently, clear steps occur from the closed state to the lowest level, which is about one-sixth of the maximum current (Fig. 1E). In all 41 patches studied, only transitions between the fully open and closed states are seen at first, followed by the invariable appearance of subconductance levels.

Records such as those shown in Fig. 1A–E strongly suggest that there are several subconductance states of these channels. Clearer examples of the six current levels recorded in several different patches are shown in Fig. 1F. The levels are always approximately an integer multiple (up to six) of one-sixth of the maximum current, although it is often difficult to differentiate between the maximum and five-sixth maximum levels because of rapid transitions between the two. Furthermore, the average current at the lowest level is often less than one-sixth

Fig. 1 A–E, Records of currents from an excised, inside-out patch of membrane following a voltage step from 0 to +57 mV (voltage of the bath solution on the cytoplasmic surface of the membrane with respect to the pipette solution). The bath and pipette contained the same solution (140 mM NaCl, 3 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM Na⁺-HEPES, pH 7.3). Upward deflections represent positive current flowing from the bath into the pipette (chloride ions moving in the opposite direction). Current was recorded on an FM tape recorder and subsequently played back through a 4-pole Bessel filter (–3 dB at 5.3 kHz), digitized at 25 µs per point and analysed by computer. Records were selected to show different current levels. Numbers on the right of each record and the broken lines show integer multiples of the lowest level seen (E); solid lines show the baseline (closed channel) current level. A, Transitions between fully open and closed levels (the commonest transitions). B, Transitions between the fully open and fourth levels (followed by a transition to the closed level) with the fifth level also present. C, Transitions between the fully open and third levels with the fourth and fifth levels also present. D, Transitions between the closed and second level with the first level also present. E, Transitions between the closed and first level. Scale bars: A, 20 pA and 100 ms; B–D, 20 pA and 20 ms; E, 7 pA and 500 ms. F, Records obtained from five different patches clearly showing maintained current levels at 1, 2, 3, 4, 5 and 6 (a–f) times the first (lowest) current level (a). The Bessel filter cutoff (–3 dB) was set in the range 0.7–3.3 kHz.



of the maximum level (see Fig. 1E), presumably because of rapid flickering between open and closed states¹⁰.

Amplitude histograms of currents recorded from individual patches also provide evidence for multiple conductance levels. Figure 2 shows an amplitude histogram of a 91-s record from the same patch as in Fig. 1. The period was chosen because it contained several lowest-level openings, including those in Fig. 1E. The histogram has peaks showing that there is a significant probability that the current could be at several levels between the fully open and closed levels. The units in the lower abscissa are one-sixth of the current between the first and last peaks. The second peak at ~5 pA gives a first-level current less than one-sixth of the maximum current level, as expected if there is rapid flickering between the first level and the closed state.

One simple model for the multiple current levels is that they are caused by contributions from six channels opening and closing independently. However, two characteristics of the currents exclude this possibility. First, following a shift in potential, some patches have conductances that are integer multiples of 400 pS and the conductance decreases in 400-pS steps. It would be a remarkable coincidence if all the 41 patches studied here contain an integer multiple of six independent channels and if the channels then dropped out in groups of six. Second, more than half of 1,000 measured transitions between clearly distinguishable levels appear to be (within less than 100 μ s) between the fully open and closed states, with no sign of an intermediate level even at 4 kHz bandwidth. If a single channel has a mean open time of 3 ms (approximately the mean duration of the first conductance level; see Table 1), the probability of six channels closing within a 100- μ s period would be $(1 - \exp(-100/3,000))^6$, that is, less than 10^{-8} . It seems very unlikely, therefore, that independent channels with a conductance of ~70 pS could produce the patterns of current levels we have observed.

Several other models to explain our observations proved unsatisfactory. For example, there could be six sequential open conformations of a single channel, each with a different conductance. This model requires the unlikely assumption that the difference in conductance is the same between each state. Moreover, we were unable to find a set of rate constants linking such states which would give rapid transitions between the closed and fully open states and still allow persistent intermediate states such as those observed. Alternatively, there could be six open states of a channel connected via a single closed state but this would suggest that different current levels should be separated by a closed level, which was seldom seen.

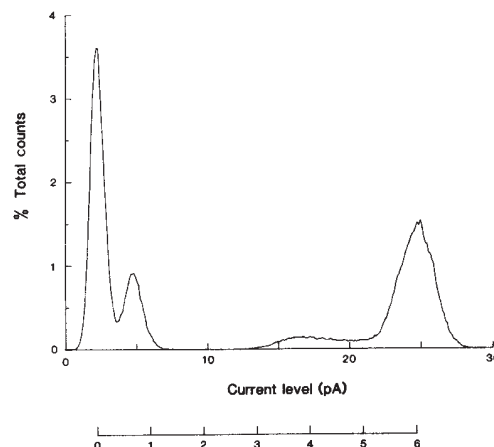


Fig. 2 Current amplitude during a 91-s recording period from the same patch as in Fig. 1A-E. The section of record was chosen for analysis because it contained examples of the first (lowest) current level and, for this reason, the histogram shows a peak at ~5 pA. Generally, only three peaks are seen (0, 4, 6), the peak for the lowest current being spread out and covering the 0, 1 and 2 current levels. The lower abscissa has units that are one-sixth of the difference between the peaks of the lowest and highest current amplitude. The probability of residing in each of the levels is given by the area under each of the peaks. The offset of the first peak from zero indicates a seal resistance of ~25 G Ω .

The appearance of seven equally spaced conductance levels together with step changes between the closed and fully open states suggests that the channel consists of six identical conducting units (co-channels) that function cooperatively to form a single channel. We believe that our results can be explained by a model in which the channel consists of a cluster of six co-channels sharing a common regulatory 'gate' that can occlude all six simultaneously. A similar model with two conducting units has been proposed previously by Miller to explain the behaviour of a small chloride channel in electroplaques⁸. It has also been suggested that the selectivity of high-conductance potassium channels arises in a similar way⁹.

To test this model, we analysed currents from two patches that exhibited fully open states at the end of recording periods of 30–100 s. Measurements were made of the time spent at each of the seven levels (0, 1, 2, 3, 4, 5 and 6 times one-sixth of maximum). To exclude noisy transitions between levels, we only accepted those levels maintained for ≥ 0.3 ms. Because of the

Table 1 Results of analysis to test for the presence of co-channels

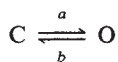
Level	No. of events	Observed open time	Observed occupancy	Open-state occupancy	Predicted occupancy	Predicted open time
0	101	2.50	0.145	—	0.001	—
1	5	3.02	0.009	0.010	0.009	3.32
2	34	4.07	0.079	0.092	0.053	3.72
3	45	4.24	0.110	0.129	0.175	4.25
4	89	5.56	0.284	0.332	0.321	4.94
5	104	6.25	0.373	0.436	0.442	6.25

Measurements of current levels lasting longer than 0.3 ms in recordings of current from two patches in which the channel continued to show full openings at the end of a recording period. Because of difficulty in distinguishing the fifth and sixth levels, the two are taken together. Observed occupancy gives the fraction of the total measurement time that a channel was at a given current level, including closed. The fraction of the total measurement time that co-channels were open (excluding the closed times that were considered to be due, primarily, to the presence of a regulatory gate) is given by open-state occupancy. Predictions of the occupancy of any of the current levels are given in the sixth column. To calculate the probabilities, it was assumed that there were six identical, independent co-channels, each with a p of 0.71. The predicted occupancies were calculated from the binomial equation $P_k = \binom{6}{k} p^k (1-p)^{6-k}$ (that is, $P_0 = (1-p)^6$, $P_1 = 6p(1-p)^5$, ..., $P_{5+6} = 6p^5(1-p) + p^6$). Deviations between predicted and observed occupancies at the lower levels are not surprising because: (1) the smaller number of events increases the measurement error; and (2) the exclusion of rapid flickering from the data would affect measured mean open time. The predicted open times were obtained from the assumption of a simple two-state model, a co-channel open probability of 0.71 and the observed open time of the 5+6 level (see text).

continuous flickering between the five-sixth and six-sixth level, the two were difficult to distinguish so were taken together and treated as a single level. The frequency of the closed state is an underestimate because we included only those times between two clearly identified open states: we excluded closed states showing rapid flickering. We have not included long closed periods (often of several seconds duration) which occur between bursts of channel openings. Results of our analysis are shown in Table 1. The fraction of the total time for which a current level was observed is given in column 4 (observed occupancy). The open-state occupancies, obtained by excluding the closed state (0 level), are also shown in Table 1.

If we assume that we are observing current flowing through six co-channels opening and closing independently (except when the channel is closed by a shared gate), the open-state occupancies should conform with a binomial distribution. Occupancies predicted for six co-channels, each with an open probability (p) of 0.71, are given in Table 1. There is good agreement between the observed and predicted open-state occupancies. Again, the discrepancy between the observed and predicted 0-level occupancies highlights the need to propose a superimposed shared gating mechanism that occludes all six co-channels at least 14.5% of the time.

Such a binomial scheme can be used to predict the probability of transitions between current levels⁸. If the behaviour of each co-channel can be described by



where C and O are the closed and open states of each co-channel, a is the opening rate and b the closing rate, it is possible to predict open times of multiple co-channel states. For example, the rate of leaving the state associated with four open co-channels is $4b + 2a$, where $4b$ is the rate for one of four open co-channels closing and $2a$ the rate for one of two closed co-channels opening. If the probability of each co-channel being open is 0.71, as before, then $a/b = p/1-p = 2.45$ and the rate of a channel leaving the four-co-channel state is $8.9b$. Rates of leaving each of the other multiple-co-channel states can be calculated in a similar way. The predicted open times (reciprocals of the rates) of each multiple-co-channel state were calculated from a value of b obtained from the mean duration of the 5+6 level (for which most data were available, see Table 1). There is clearly good agreement between observed open and predicted open times (Table 1).

Thus, evidence for our co-channel model is: (1) channels of high conductance that show seven equally spaced conductance levels; (2) a high percentage of rapid transitions between closed and fully open states; (3) open-state probabilities and open times consistent with the existence of six identical, independent co-channels; and (4) a closed-state probability and closed time greater than expected from six independent co-channels.

This kind of channel behaviour is probably not confined to pulmonary alveolar cells. Records of currents from high-conductance chloride channels in a variety of tissues⁵⁻⁷ show subconductance states and transitions similar to those described here. Furthermore, multiple conductance levels between fully open and closed levels have been noted in other large-conductance channels¹¹⁻¹³ and single subconductance states have been seen in smaller channels¹⁻³. Indeed, it has been reported recently that a potassium channel in an *Aplysia* neurone¹⁴ displays both equally spaced conductance levels and rapid transitions between fully open and closed states. Such observations raise the possibility that a mechanism involving clusters of co-channels with a shared gate functions in a wide variety of channels in other preparations. If so, the phenomenon is important in channel assembly and function and must be taken into account during analysis of single-channel currents and in the formulation of theories of ion permeation through membranes.

After submission of this paper, a high-conductance chloride channel with 17 conductance levels in a molluscan neurone has been reported¹⁵ and we have observed channels with more than seven current levels.

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Permanent distortion of positional system of *Xenopus* embryo by brief early perturbation in gravity

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The formation of a body plan from an initially radially symmetrical egg during animal development is presumed to involve a 'positional system'^{1,2} and a subsequent mechanism of local response to position values provided by this system. Early gene products intimately connected with the latter response mechanism have been identified in *Drosophila*³⁻⁵, and because these share conserved sequences with possible counterparts in vertebrates, there is renewed interest in understanding the physiological nature of the positional system itself in a vertebrate embryo. In the frog *Xenopus*, body position value appears to be specified in outline across much of the egg material by stages comprising a few cells, after only ~2 h of development⁶⁻¹⁰. This is already suggestive of a structural or mechanical recording system rather than a diffusion-controlled gradient. I describe here an experiment aimed at perturbing the positional system by causing gravity-driven rearrangements within eggs which conflict with their own, self-organizing rearrangements near the time of first cleavage. The system appears to retain its full regulatory properties during only a brief time interval, so that records of positional profiles disturbed at the close of that interval are permanent, and give rise to systematically abnormal body patterns in otherwise healthy larvae. The results are inconsistent with the notion that *Xenopus* primary pattern results from a set of determinant 'plasms' in the egg, or from a mechanism dominated by long-range diffusion of molecules^{11,12}.

The direct action of the positional system occurs mainly in the yolk, endodermal part of the egg. Coordinated mesodermal and ectodermal body contributions are set up later by appropriately balanced spatial patterns of inductive activity emanating from the endoderm¹³⁻¹⁶. The deviant bodies that I shall describe here are detectable on external form alone (top row, Fig. 1), but I have also analysed the mesoderm, whose anatomy is sufficiently differentiated at early larval stages. After 48 h of development (stage 29-30; ref. 17), all mesoderm cells can be scored histologically as members of the different axial pattern elements (see Fig. 2), but there has been little growth to obscure the proportions in which the tissue was originally partitioned

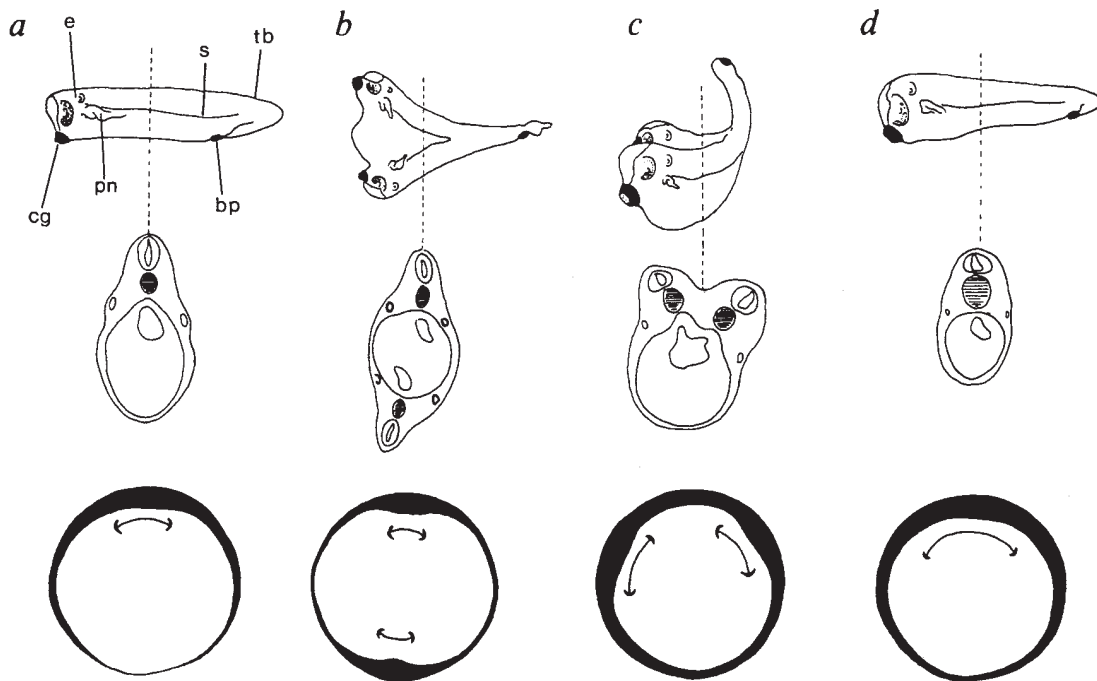


Fig. 1 Twinned and imbalanced body patterns seen after tilting eggs in gravity, and a suggested interpretation. The top row shows camera lucida sketches of the normal body form (*a*) and of three typical experimentally produced body forms (*b–d*) at the larval stage used for pattern analysis. *b*, A twinned pattern with approximately equal and opposite partial axes and moderate deficiency of posterior pattern. *c*, A twinned pattern with adjacent and massively constructed partial axes but an extreme deficiency of posterior pattern. *d*, An imbalanced single pattern with systematic over-allocation to dorsal and anterior structure at the expense of posterior structure. The second row shows schematic transverse sections of each body form, at approximately 8th somite level. Neural tubes, notochords (shaded) and the yolkly gut mass with a single or double archenteric cavity are shown. Note the relative sizes of the notochord in cross-section. The third row shows corresponding egg outlines in the horizontal plane relative to normal gravity orientation. The thickness of the outline represents the profile around the egg yielding each body form, with respect to levels of activation, or 'body position values', recorded by the early-acting positional system. With the normal, complete profile (*a*), the mes-endodermal body plan is built essentially from head plus notochord towards tail, around the egg meridians from top to bottom of the outline, the position of sperm entry being near the bottom. The separate internal arc opposite the thickest part of the outline represents arbitrarily the normal extent of an egg sector specified for allocation to notochord production. The outlines of *b* and *c* suggest how, when two such peaks of activation arise in one egg after early perturbation of the plasm shifts, interactive effects due to their relative positioning may influence greatly the profile attained by each, and hence the relative amount of the system allocated to notochord and anterior structure. There is a corresponding variable sacrifice of the posterior pattern complement. Such variation is observed (see text). Outline *d*, corresponding to a single but incorrectly proportioned axial pattern, is perhaps an extreme version of type *c* with partial regulation fusing the original centres of activation. Externally visible larval features: e, eyecup; cg, cement gland; pn, pronephros; s, line of lateral edges of somites; bp, proctodaeum (original blastopore); tb, tailbud.

into founder territories for these structures¹⁸. Mesodermal pattern balance in individuals is therefore assayed by scoring the nuclei encountered on every fifth 7- μ m section of the transverse series, and sampling $\sim 10^4$ cells (or one-third of the total when nuclear size and section thickness are properly considered¹⁹).

The mesodermal pattern is highly consistent among individual normal embryos, and is subtle in the sense that characteristic proportions of the totals of somite and lateral plate cells are allocated to these structures at each level in the axis⁹. This feature, a measure of the relative 'head-' or 'tail-heaviness' of tissue distribution along the normal series of somite segments, can be quantified as the 'somite index' (see Table 1), which has a certain range of normal variation.

My attempt to force the system outside its normally regulated performance was based on recently published work concerning mechanical aspects of the pre-cleavage events^{6,7,20–22}. After fertilization, a system of spontaneous sliding displacements between deep egg regions and the surface structure, driven in relation to the position at which the sperm aster is developing, normally orientates and activates the development of the body from the egg's substance. Any artificially maintained tilt of the eggs away from their natural orientation in gravity, at times when their self-generated movements are occurring, can override the orientating effect of the sperm. Normal patterns then develop, but are symmetrical around that plane in which gravity

has caused the egg contents to rearrange. There is much to suggest^{10,23} that the total set of movements within the normal egg, more than merely orientating its pattern formation, is quantitatively related to normal completeness and proportioning of the pattern itself.

Towards the end of the pre-cleavage interval, progressive 'gelling' of some egg component appears to record unalterably the net results of all preceding reorganizations in setting up a pattern, whether these were dominated by the natural mechanism or by artificial tilt. Therefore, tilting in gravity at a late stage in this sensitive period, in directions calculated to conflict widely with the system of activation orientated by sperm entry in each egg, might lead to the recording of positional profiles different from those resulting from either natural or imposed systems when acting alone. Such a regime tilt through 70–100° in planes that raised the sperm entry point (s.e.p.) very high in gravity, beginning at 65–85% of the time from fertilization to first cleavage, was accordingly applied to experimental populations of eggs from synchronously fertilized clutches. At the chosen temperature (19 °C), this involved tilting at between 65 and 85 min after fertilization. The eggs were released to orientate freely in gravity at the 8-cell stage after 1 h. Control eggs were subjected to identical conditions except that they were not tilted (for production and handling of eggs in such experiments, see refs 8, 20, 21).

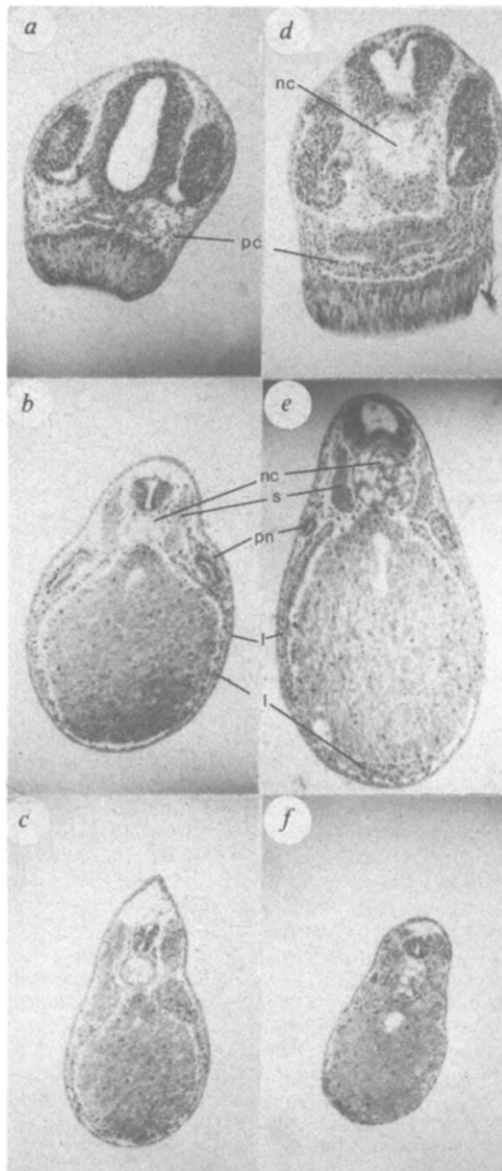


Fig. 2 Internal structure of normal and pattern-imbalanced larvae. *a–c*, Sections taken at different levels in control larva at stage 29; *d–f*, equivalent sections in a sibling morphologically 'disturbed' after pre-cleavage tilt. *a, d*, Level of forebrain, eyecups, ventral prechordal mesoderm and cement gland. Note the massive size of the latter two regions in the disturbed case, and the intrusion of the massive notochord cross-section into this anterior level. *b, e*, Level of highest cell count in pronephros (approximately opposite somite 5). Note the greater cross-sectional area of the gut mass, under-allocation to pronephros, the massive notochord and wide floor plate of the neural tube, and the large lateral plate population in the disturbed case. *c, f*, Level just anterior to the proctodaeum (blastopore). Note the small cross-section and diminished scale of construction of the entire mesoderm in the disturbed case. Only at this level does the cell number in the notochord cross-section return to normal values. nc, Notochord; pc, prechordal mesoderm; pn, pronephros; l, lateral plate; s, somite.

Six populations of ~80 eggs each were subjected to the experimental regime, with tilting of each individual in sequence being completed across the 20-min period. Synchronously fertilized siblings in similar numbers were used as controls for each population. After 2 days, five of the experimental populations included instances of twinned or mirror-duplicated pattern and of a characteristic, 'imbalanced' single body pattern, in normally healthy larvae. The single-body pattern involved large ventral

head and anterior trunk regions but small, slender posterior parts (Fig. 1*d*). There were between 2 and 6 twins per population (26 in total), in contrast to only one among the 450 controls. Affected populations showed between 4 and 12 imbalanced single bodies (39 in all), whereas none was seen either among the controls, in three populations where s.e.p. up tilt was imposed before the midpoint of pre-cleavage, or in three populations where this tilt was applied for 1 h between the 4- and 32-cell stages.

Larvae showing the externally deviant morphology all had quite abnormal balances of mesoderm pattern in relation to all the controls, or to apparently normal members of their experimental batches. This is illustrated for two typical sets of such bodies in Table 1. That the control/experimental differences shown in Figs 1*a, d* (camera lucida) and 2*a–f* (transverse section) may appear undramatic to the non-specialist, is a measure of the precision of performance of the normal pattern formation under study. As in previous work of this kind in *Xenopus*, an abnormal pattern *per se* is not associated with abnormal numbers of mesoderm cells¹⁸. Internal features of the syndrome (for 20 abnormal mesoderms analysed completely) are as follows: (1) The cellular allocation to notochord is 1.5–2.5 times normal, the excess being concentrated towards the anterior of the axis. (2) The allocation to the ventral pre-chordal structure, and particularly the anterior lateral plate, is enhanced, while that to the total somite is usually somewhat reduced. The pre-chordal imbalance is reflected in the deep, broad head and excessive area of cement gland induced in the head ectoderm. (3) Although the total somite segment number is correct, the axis is anterior-heavy. While the slender anterior somites are allocated at least their normal cell numbers, the cross-sections of those in the trunk and tail are progressively reduced below the norm (Fig. 2*b, c, e, f*; see also Table 1). The posterior lateral plate and tailbud tissues are also under-represented in relation to the massive 'chest' tissue. (4) The allocation to the pronephros is frequently abnormally small, down to less than half the normal 6–9% of mesoderm (Fig. 2*b, e*), or is even absent.

The twinned bodies turn out to represent opposed pairs of partial body patterns, the regions represented in each axis being the entire notochord and those other parts of the plan that are over-represented in the imbalanced singles. The pronephros and those parts of the somite pattern normally contributed from near the s.e.p. meridians, are reduced or absent. Two features of the variation seen among these twin patterns are particularly informative concerning the dynamical properties of the early positional systems. The first concerns notochord allocation. In many cases the two notochords lie relatively close together, suggesting that the centres of 'activation' of two positional systems arising in the egg were situated considerably less than 180° apart. There is then normal or supranormal cellular allocation to each notochord, and thus twice or more the normal allocation to this structure (Fig. 1*c*, second and third rows). In other cases, where the midlines of the twinned system are equal and opposed in the egg, there is a diminished allocation to each notochord such that the total allocation is little above normal (Fig. 1*b*). The second noteworthy feature (not represented in Fig. 1) is that one or both patterns may be locally incomplete at the extremities of the head and anterior notochord, and there may be a large asymmetry in partitioning of the total cell population to smaller and larger, complete or anteriorly incomplete patterns.

Figure 1 (third line) offers an interpretation of the patterns, in terms of profiles with respect to some variable that could be termed position value for pattern contribution, encoded into the structure around the egg margin. All the imbalances produced are systematic over-allocations of egg meridians to those position values which are normally specified relatively far from the point of sperm entry, and are associated with the fastest developing meridians of the egg and gastrula.

It is clear that abnormal profiles of the primary positional variable, recorded at an essentially pre-cellularized stage, can

be maintained for many hours and cell cycles; they are ultimately transmitted from endodermal regions as aberrations in the balance of mesoderm specification. Positional systems that are incomplete in the opposite sense—that is, lacking dorso-anterior values entirely—show similar stability from egg to larva^{6,22,23}. Such behaviour, and the rapidity with which the system is set up, is very different from what would be expected from a reaction/diffusion or source/diffusion morphogen landscape, with substance concentration being registered as the position value^{11,12}. The various stably maintained asymmetries that are seen in twinned positional profiles also deviate from the behaviour expected of reaction/diffusion. On the other hand, the fact that simple mechanical manipulation can alter drastically the relative allocations to different parts of the pattern in the egg as a whole, is scarcely compatible with the idea that a set of determinant plasmids is simply deployed by the pre-cleavage events.

Table 1 Pattern balance in sibling sets after exposure of eggs to an experimental regime of gravity re-orientation

		% Mesoderm cells in:				Somite ratio	
		NC	S	PN	LPL		
Set 1							
Controls (no tilt s.e.p. up)	{	1	5.6	43.4	6.0	45.0	3.4
		2	6.3	47.1	5.3	41.3	3.1
		3	5.7	45.6	5.5	43.2	3.5
Controls (tilt, but normal morphology)	{	4	5.7	46.7	5.0	42.6	2.9
		5	6.0	44.1	5.6	44.3	3.5
Experimentals (tilt, then deviant morphology)	{	1	8.9	37.4	3.2	50.5	2.1
		2	9.8	44.0	4.3	41.0	2.4
		3	10.1	38.6	3.9	47.4	1.7
		4	11.4	35.0	2.1	51.5	1.0
		5	9.5	39.5	3.8	49.2	1.8
Set 2							
Controls (no tilt s.e.p. up)	{	1	5.6	44.2	5.7	44.5	2.9
		2	5.8	47.4	6.6	40.2	2.8
		3	6.0	41.9	7.2	44.9	2.5
Controls (tilt, but normal morphology)	{	4	4.9	44.5	5.4	45.2	2.7
		5	5.4	46.2	6.1	42.3	2.4
Experimentals (tilt, then deviant morphology)	{	1	11.0	32.5	3.5	53.0	1.5
		2	14.6	38.5	1.5	45.4	0.9
		3	8.5	37.7	5.0	48.8	2.0
		4	9.6	32.7	6.2	51.5	2.0

Somite ratio is the mean maximum number of nuclei seen in transverse sections through somites 14–16 inclusive, divided by the equivalent value for sections of somites 4–6 inclusive. This ratio is indicative of the way in which mesoderm has been allocated to form the pattern of different axial levels, and values between 2.0 and 3.5 characterize the normal embryos from various ovulations. S, somite; PN, pronephros; NC, notochord; LPL, lateral plate.

Mechanochemical models of pattern production (see, for example, ref. 24), invoking the generation of local variations in some non-fluid aspect of the egg's structure that are then inherited by the blastomeres, may have relevance to this relatively small yet rapidly developing vertebrate egg. A long-term research goal must be the elucidation of ways in which structural or physiological modulation of early cellular material can stably affect the local developmental rate^{6,7,23} and, ultimately, set position-specific modes of transcription in nuclei. Possible reasons for the previous report of prolonged regulatory ability for body pattern formation, following other operations carried out on *Xenopus* blastula stages²⁵, have been discussed elsewhere⁸.

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Human T-cell clones recognize a major *M. leprae* protein antigen expressed in *E. coli*

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Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. As with other intracellular parasites, protective immunity is dependent on T cells and cell-mediated immunity¹. In animal models, immunization with killed armadillo-derived *M. leprae* elicits strong T-cell responses, delayed-type hypersensitivity and protection against viable challenge^{2–5}. We have recently shown that killed *M. leprae* can induce delayed-type hypersensitivity in healthy human volunteers⁶. Identification of the *M. leprae* antigens that are recognized by T cells and may be involved in protection has been hampered by the inability to cultivate the organism *in vitro* and by difficulties in antigen purification from limited quantities of armadillo-derived bacillus. Because genes for the major protein antigens of *M. leprae* as seen by mouse monoclonal antibodies have been isolated^{7,8}, it has become possible to test whether these individual antigens are recognized by T cells. We screened crude λ gt10 phage lysates of *Escherichia coli* containing individual *M. leprae* antigens using *M. leprae*-specific T-cell clones isolated from *M. leprae*-vaccinated volunteers. Using this method, we find that nearly half of the *M. leprae*-specific T-cell clones are stimulated to proliferate by lysates containing an epitope of a *M. leprae* protein of relative molecular mass 18,000 (18K).

Healthy human volunteers vaccinated with killed *M. leprae* were sensitized to soluble *M. leprae* antigens⁶. Forty-two T-cell

Table 1 Antigen reactivity and surface phenotype of *M. leprae*-specific and cross-reactive T-cell clones

Clones:	AF/6C8	AF/5C10	ATT 1/10B	ATT 2/5A	ATT 2/4F	ATT 6/10F	ATT 5/1D	NHH 2/2F	NHH 1/E7	NHH 5/1D	WR 7/F6	ATT 2/6E	ATT 4/1F	ATT 4/4C
Antigens														
Control	120	150	139	561	435	291	149	380	251	139	564	612	409	572
Soluble <i>M. leprae</i> (CD45)*	<u>5,590</u>	<u>22,065</u>	<u>5,773</u>	<u>4,027</u>	<u>5,300</u>	<u>1,615</u>	<u>6,068</u>	<u>9,585</u>	<u>3,607</u>	<u>4,858</u>	<u>29,065</u>	<u>15,177</u>	<u>5,424</u>	<u>17,128</u>
PPD	374	261	257	696	589	140	142	681	318	200	322	<u>23,586</u>	<u>2,919</u>	<u>6,489</u>
BCG	108	237	270	436	278	298	128	271	178	287	479	ND	<u>16,114</u>	ND
SPA 30†	<u>39,562</u>	<u>26,638</u>	<u>3,441</u>	<u>3,194</u>	ND	<u>1,273</u>	ND	<u>4,658</u>	ND	<u>1,943</u>	<u>28,982</u>	ND	ND	ND
Whole <i>M. leprae</i> (CD45)	<u>3,565</u>	<u>17,957</u>	<u>7,076</u>	<u>3,334</u>	ND	<u>2,010</u>	ND	<u>10,471</u>	<u>3,205</u>	<u>5,774</u>	ND	ND	ND	ND
Human <i>M. leprae</i>	<u>4,177</u>	<u>8,466</u>	<u>2,558</u>	<u>2,605</u>	ND	<u>1,525</u>	ND	<u>8,311</u>	<u>2,810</u>	<u>4,681</u>	<u>4,102</u>	ND	ND	ND
Armado protein	115	273	106	223	ND	183	ND	295	224	ND	ND	ND	ND	ND
<i>M. tuberculosis</i> (H37Rv)	ND	171	125	710	ND	117	ND	259	488	221	ND	<u>11,982</u>	ND	<u>16,644</u>
<i>M. vaccae</i>	ND	754	103	169	ND	183	ND	78	96	186	ND	809	ND	<u>3,740</u>
Nonchromogenin	325	277	162	304	495	115	221	134	146	337	<u>5,363</u>	616	<u>2,092</u>	645
Vaccin	325	422	125	254	469	135	356	107	498	296	109	864	917	1,411
Xenopin	131	375	140	321	417	87	246	146	99	326	332	1,009	<u>5,818</u>	<u>5,890</u>
Schrofulin	520	435	<u>2,526</u>	<u>1,453</u>	<u>2,694</u>	<u>1,862</u>	522	<u>2,985</u>	281	478	126	<u>2,452</u>	<u>3,165</u>	<u>2,748</u>
Kansasin	261	89	267	597	564	125	171	206	81	389	118	<u>3,492</u>	<u>5,266</u>	<u>3,690</u>
Tuberculin	215	550	89	527	ND	153	ND	585	196	419	120	<u>1,806</u>	ND	<u>2,003</u>
Duvalin	358	498	112	317	272	145	263	183	192	251	516	1,430	<u>2,164</u>	820
Neaurumin	806	806	45	255	653	316	448	106	133	749	449	520	319	479
Flavescin	243	142	313	414	420	569	631	58	63	ND	345	900	<u>2,050</u>	571
Aviumin A	192	324	140	1,169	147	98	294	137	164	ND	226	<u>2,568</u>	<u>2,986</u>	<u>2,659</u>
Aviumin C	295	376	505	566	ND	173	ND	202	420	ND	299	631	ND	485
Glaxo BCG sonicate	540	337	176	342	ND	210	ND	720	402	ND	166	<u>12,009</u>	ND	<u>14,925</u>
Phenotype CD4	+	+	+	+	+	+	+	+	+	ND	+	+	+	+
CD8	-	-	-	-	-	-	-	-	-	ND	-	-	-	-

Peripheral blood mononuclear cells (PBMC) were separated from five healthy volunteers vaccinated with 1.5×10^8 or 4.5×10^8 killed *M. leprae*⁶. 20×10^6 PBMC (2×10^6 cells ml^{-1}) in complete medium (RPMI 1640+15% human AB serum+1% penicillin/streptomycin) were cultured with $10 \mu\text{g ml}^{-1}$ soluble *M. leprae* antigens (Batch CD45 from R. J. W. Rees through WHO/IMMLEP Bank) in 25-cm² flasks (Costar). Flasks were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air. On day 6, cells were washed and recultured with fresh autologous irradiated (2,500 rad) feeder cells+antigen. On day 9, viable cells were recovered on Lymphoprep gradient and cloned in 60-well Microtest plates (Falcon) by limiting dilution¹¹. After 7–10 days, cells from growing wells were transferred to 96-well flat-bottom plates (Nunc) with fresh autologous feeders+antigen+recombinant interleukin-2 (IL-2; Cetus). After further incubation at 37 °C for 7–10 days, growing cultures were transferred to 24-well plates (Nunc) with $1 \times 10^6 \text{ ml}^{-1}$ autologous irradiated PBMC as feeder+ $10 \mu\text{g ml}^{-1}$ soluble antigens of sonicated *M. leprae*+ 100 U ml^{-1} recombinant IL-2. Recombinant IL-2 alone was added on day 4. The cycle was repeated every seventh day. When T cells reached $1-2 \times 10^6 \text{ ml}^{-1}$, they were subcultured at 2×10^5 cells per well. After 4–8 weeks of cloning, sufficient cells were available for antigen testing. To test antigen reactivity of the clones in a proliferative assay, adherent cells from 10^5 irradiated PBMC were distributed into each well of 96-well flat-bottom trays and used as antigen-presenting cells; 10^4 cloned T cells and antigens at optimal concentration were added. Antigens were either whole bacilli or soluble sonicates of mycobacteria¹². Cells were incubated for 48–72 h at 37 °C in 5% CO₂ and 95% air. At 4 h before collection, cultures were pulsed with 0.045 MBq ³H-thymidine (specific activity $185 \times 10^3 \text{ MBq mol}^{-1}$), incorporated radioactivity was measured¹³ and median values of counts per minute (c.p.m.) determined. A clone was considered to be proliferating in response to a given antigen when incorporated c.p.m. in cultures with antigen minus c.p.m. in cultures without antigen was $\geq 1,000$ and $T/C > 2$ (such values in the table are underlined) where $T/C = (\text{c.p.m. in cultures with antigen})/(\text{c.p.m. in cultures without antigen})$. Phenotypes of the T cells (CD4 or CD8) are also listed.

* Soluble *M. leprae* was an ultrasonicated and Millipore membrane (0.22 μm)-filtered preparation of whole bacilli batch CD45 (from R. J. W. Rees).

† SPA 30 was the filtrate of soluble *M. leprae* filtered through Amicon ultramembranes with 30,000 relative molecular moles cut-off (from J. Convit).

clones raised against *M. leprae* soluble extracts were established from five volunteers. These cells were screened for reactivity to antigens prepared from *M. leprae*, BCG, *Mycobacterium tuberculosis*, PPD (purified protein derivative) and other mycobacteria. Eleven of the 42 T-cell clones reacted with *M. leprae* antigens derived from humans or armadillos, but not with BCG or PPD; the responses of these 11 clones to various antigen preparations are shown in Table 1. The response to antigen preparations from other mycobacteria was absent or low, restricted to *Mycobacterium scrofulaceum* (5 clones) and *Mycobacterium nonchromogenicum* (1 clone). The responses of three T-cell clones that exhibit broad mycobacterial cross-reactivity (ATT 2/6E, ATT 4/1F, and ATT 4/4C) are included for comparison.

Five abundant *M. leprae* proteins with relative molecular masses of 65K, 36K, 28K, 18K and 12K have been identified through the use of monoclonal antibodies^{7–9}. Each of the genes encoding these proteins has been cloned in *E. coli* Y1089 (ref. 7). Lysogens of λ gtlI recombinant phage carrying each of the five *M. leprae* genes were constructed and used to express the antigen of interest in a crude lysate¹⁰. Similar amounts of *M. leprae* antigen were produced in each of the five lysates. The crude lysates were tested for their ability to stimulate 10 of the *M. leprae*-specific T-cell clones (Table 2); 5 of 10 *M. leprae*-specific T-cell clones responded to *E. coli* lysates containing antigen expressed from the recombinant phage Y3179 (ref. 7). Four of these reactive T-cell clones were established from one

volunteer, the other came from a second volunteer. The pattern of reactivity of the T-cell clones suggests that the λ gtlI phage Y3179 expresses an *M. leprae* epitope that is absent in BCG, *M. tuberculosis* and other cultivable mycobacteria, but is shared with *M. scrofulaceum*.

The λ gtlI clone Y3179 expresses an antigenic determinant

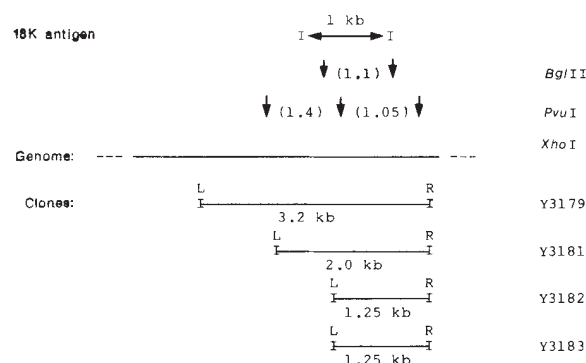


Fig. 1 Restriction endonuclease mapping and *M. leprae* DNA size of recombinant phage Y3179, Y3181, Y3182 and Y3183 expressing epitope of an 18K protein antigen recognized by a single *M. leprae*-specific monoclonal antibody L.7.17^{7–9}. Arrows ($\downarrow$) show the sites of restriction endonuclease action, and the size of *M. leprae* DNA thus produced is given in parentheses.

Table 2 Proliferation of T-cell clones in response to the recombinant *M. leprae* antigens from phage vector λ gtlI expressed in *E. coli*

T-cell clones	—*	Soluble <i>M. leprae</i> (CD45)	λ gtlI control	Lysates from <i>E. coli</i> infected with				
				Y3178	Y3180	Y3164	Y3179	Y3184
				<i>M. leprae</i> specific antigens expressed				
				64K	36K	28K	18K	12K
Specific clones								
AF/6C8	201	35,331	345	328	51	160	169	100
AF/5C10	271	5,718	579	259	98	627	387	127
ATT 1/10B	554	29,379	326	424	1,009	295	2,926	384
ATT 2/5A	320	24,532	550	133	187	146	8,225	175
ATT 2/4F		31,505	218	115	304	136	11,382	165
ATT 5/1D	318	23,411	275	181	212	422	101	158
ATT 6/10F	110	29,928	215	114	106	117	8,746	156
NHH 2/2F	91	23,761	109	201	161	76	10,389	101
NHH 1/E7	148	4,461	350	143	167	209	208	246
WR 7/F6	530	12,291	202	186	170	131	132	71
Cross-reactive clones								
ATT 4/1F	808	30,246	858	496	455	691	430	680
ATT 2/6E	803	25,626	876	1,366	965	1,071	634	691
ATT 4/4C	511	6,953	779	734	548	596	643	592

Crude *E. coli* lysates containing individual *M. leprae* antigens were prepared by inducing λ gtlI recombinant lysogens of the strain Y1089¹⁰. Single colonies of recombinant lysogens were inoculated into 100 ml of LB medium (pH 7.5) and cultures were incubated at 32 °C. When cells had grown to $A_{600} = 0.5$, cultures were transferred to 45 °C and incubated for 20 min. Isopropyl β -D-thiogalactopyranoside (IPTG) was added to 10 mM and the cultures were further incubated at 38 °C for 1 h. All incubations were carried out with good aeration. The cell pellet obtained after centrifugation was resuspended in saline 1/20 of original culture volume and frozen immediately in liquid nitrogen. Thawing of frozen cells resulted in lysis of induced lysogen. To reduce viscosity, crude lysates were briefly sonicated (3×10 s) on ice, and then sterilized by Millipore filtration. Lysates were then frozen at -70 °C in aliquots until used. Antigenic activity was lost rapidly on storage at +4 °C, so new vials were used when stimulating T-cell clones. Expression was confirmed by probing crude lysates with the appropriate monoclonal antibodies^{7,8}. T-cell clones were tested with different doses of crude lysates as described for other antigens in Table 1. Results are given for $10 \mu\text{g ml}^{-1}$ of crude lysate and expressed as in Table 1.

* Control cultures without antigen.

Table 3 Proliferation of T-cell clones to Y3179 and other recombinant antigens recognized by L7.15 antibody

T-cell clones	—*	<i>E. coli</i> lysate containing expressed antigens from:							
		Y3179		Y3181		Y3182		Y3183	
		10 μg	50 μg	10 μg	50 μg	10 μg	50 μg	10 μg	50 μg
2/4F	541	22,370	31,728	2,839	6,425	751	2,580	753	2,527
2/5A	445	21,266	16,224	2,510	10,368	1,178	3,009	1,290	5,727
6/10F	471	12,415	11,638	1,873	6,592	1,167	3,016	1,625	5,073

Crude lysates containing antigens expressed by phage Y3179, Y3181, Y3182 and Y3183 were prepared as described for Table 2. Responses of the T-cell clones to two lysate concentrations ($10 \mu\text{g ml}^{-1}$ and $50 \mu\text{g ml}^{-1}$) are given as in Table 1.

* Control cultures without antigen.

of an 18K *M. leprae* protein recognized by the monoclonal antibody L7.15 (refs 7, 8). Y3179 contains 3.2 kilobases (kb) of *M. leprae* DNA, sufficient to encode additional polypeptides that might be fortuitously expressed in the Y3179 lysate and which could conceivably be responsible for stimulation of the T-cell clones. However, two lines of evidence favour the idea that some portion of the 18K protein is responsible for T-cell stimulation. Additional λ gtlI clones containing smaller inserts and sharing only 1.25 kb of DNA produce the epitope recognized by L7.15 (Fig. 1); *E. coli* lysates of these phage stimulate the same T-cell clones that respond to Y3179 (Table 3). More importantly, while most *M. leprae* proteins exhibit immunological cross-reactivity with proteins from other mycobacteria, the L7.15 epitope is specific to *M. leprae*^{8,9}, as is the response of the five *M. leprae*-specific T-cell clones. The precise nature of the stimulatory epitope is being investigated.

These results establish that vaccination of normal volunteers with killed *M. leprae* induced a population of CD4-positive T cells that could be cloned *in vitro* and which exhibited *M. leprae*-specific reactivity. In addition, T-cell clones from two volunteers were capable of responding to an *M. leprae* antigen expressed in crude λ gtlI phage lysates of *E. coli*. This and possibly other *M. leprae* antigens recognized by T-cell clones may be relevant to protection against leprosy.

The methods used here should be generally applicable to the identification of parasite polypeptide antigens recognized by T cells. While there is a high background of nonspecific reactivity of normal peripheral blood lymphocytes to crude phage lysates, antigen-specific T-cell clones produce a relatively minor background response, making it possible to test their reactivity to individual antigens in crude lysates.

For leprosy, these methods should facilitate further identification and characterization of *M. leprae* antigens recognized by T cells. Once these antigens are purified, it should be possible to evaluate their relative importance to the T-cell repertoire of *M. leprae*-immune individuals and to examine their role in protection against infection.

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Mycobacterium leprae-specific protein antigens defined by cloned human helper T cells

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Leprosy displays a remarkable spectrum of symptoms correlating with the T-cell-mediated immune reactivity of the host against the causative organism, *Mycobacterium leprae*¹. At one pole of this spectrum are lepromatous leprosy patients showing a *M. leprae*-specific T-cell unresponsiveness²; at the other are tuberculoid leprosy patients displaying both acquired immunity and delayed-type hypersensitivity against *M. leprae* which are thought to be conferred by helper T (T_H) cells³⁻⁵. Because well-defined *M. leprae* antigens are crucial for the prevention and control of leprosy^{1,6,7}, we have cloned *M. leprae*-reactive T cells (TLC) of the helper phenotype from a tuberculoid leprosy patient. As reported here, these TLC show an unexpected diversity in the recognition of *M. leprae* and related mycobacteria, which is different from that exhibited by monoclonal antibodies^{8,9}. Half of these TLC are completely or almost *M. leprae*-specific, whereas the other half are cross-reactive with most or all other mycobacteria. A *M. leprae* protein of relative molecular mass (*M*_r) 36,000 (36K) defined by a *M. leprae*-specific monoclonal antibody^{7,8,10} stimulates 4 out of 6 TLC tested. Each of these TLC recognizes a different antigenic determinant, one of which is *M. leprae*-specific. The previous paper describes other *M. leprae*-specific T-cell clones half of which recognize an epitope on a *M. leprae* protein of *M*_r 18 K.

Whereas the *M. leprae*-specific unresponsiveness in lepromatous leprosy patients may be triggered by specific immunosuppression-inducing determinants on the bacillus¹¹, virtually nothing is known about the antigens recognized by T_H cells responsible for the cell-mediated immune reactivity against *M. leprae* in tuberculoid leprosy patients. To study the antigenic determinants involved in activating these T cells in more detail, we have cloned *M. leprae*-reactive T cells of a tuberculoid leprosy patient.

Peripheral blood mononuclear cells of a tuberculoid leprosy patient were restimulated *in vitro* with *M. leprae* antigen, cloned and propagated as described elsewhere¹². Autologous Epstein-Barr virus (EBV)-transformed B cells (EBV-BC) were used as antigen-presenting cells (APC), as evidence suggests that these cells can effectively present *M. leprae* antigens to primed T cells (for review see ref. 13). One advantage of using EBV-BC as APC is that such cells provide a continuous and unlimited source

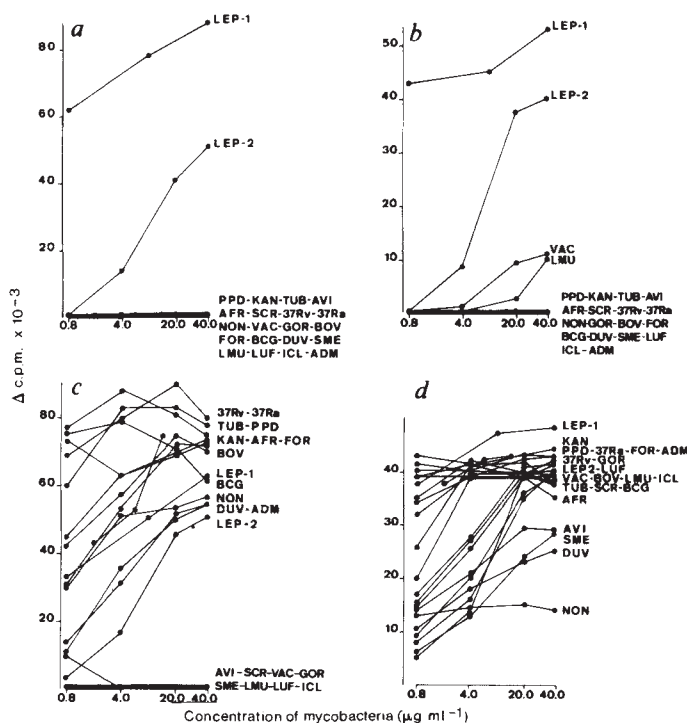


Fig. 1 Antigen specificity of *M. leprae*-reactive cloned T cells. Mycobacterial antigens were presented via autologous or HLA class II-compatible APC to 23 different *M. leprae*-reactive TLC from a tuberculoid leprosy patient. Four different patterns of reactivity were observed, and a representative example of each pattern is shown (*a-d*) (pattern *a*: *n* = 6, *b*: *n* = 5, *c*: *n* = 2, *d*: *n* = 10). The antigens tested were: LEP-1, Dharmendra lepromin, consisting of bacilli isolated from human lepromas; LEP-2, armadillo-derived *M. leprae*; PPD, purified protein derivative of *M. tuberculosis* (obtained from Statens Serum Institute, Copenhagen); KAN, *M. kansasii*; TUB, *M. tuberculosis*; AVI, *M. avium*; AFR, *M. africanum*; SCR, *M. scrofulaceum*; 37Rv, *M. tuberculosis* H37Rv; 37Ra, *M. tuberculosis* H37Ra; NON, *M. non-chromogenicum*; VAC, *M. vaccae*; GOR, *M. goodii*; BOV, *M. bovis*; FOR, *M. fortuitum*; BCG, *M. bovis* BCG; DUV, *M. duvalii*; SME, *M. smegmatis*; LMU, *M. lepraemurium*; LUF, *M. lufu*; ICL, *M. avium* intracellulare; ADM, armadillo-derived aspecific mycobacteria. Dharmendra lepromin was tested in dilutions of 1:240, 1:120 and 1:60. PPD was tested at concentrations of 1.7, 5.0 and 16.7 $\mu\text{g ml}^{-1}$. All other mycobacteria had been ultrasonicated as described in ref. 21, and were tested at 0.8, 4.0, 20.0 and 40.0 $\mu\text{g ml}^{-1}$. Results are mean $\Delta\text{c.p.m.} \times 10^{-3}$ (^3H -thymidine incorporation) of duplicate cultures (standard deviations mostly <10%), as assessed by subtracting background proliferation in the absence of antigen from responses in the presence of antigen.

Methods. Antigen reactivation, cloning, expansion and proliferative assays were performed as described elsewhere¹². Briefly, peripheral blood mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation and restimulated *in vitro* with an optimal concentration of Dharmendra lepromin (1:120-1:60) in 24-well tissue culture plates. T-cell blasts, enriched for by Percoll density centrifugation, were then cloned by limiting dilution on a feeder cell mixture consisting of irradiated autologous EBV-BC (10^5 cells ml^{-1}), peripheral blood mononuclear cells from 3-4 random donors (10^6 cells ml^{-1}), and Dharmendra lepromin (1:120-1:60). Of the original 295 microwells seeded, 56 contained growing cultures, and 34 of these showed proliferative responses. Growing cultures were restimulated once a week with the feeder mixture, supplemented with Leuko Agglutinin A (1 $\mu\text{g ml}^{-1}$) (Pharmacia) and expanded in the presence of interleukin-2-containing supernatants. In proliferative assays, 10^4 TLC, 5×10^4 irradiated autologous or HLA class II-compatible peripheral blood mononuclear cells as APC, and antigen were co-cultured in flat-bottomed 96-well microtitre plates (Greiner, FRG). After 72 h, 1.0 μCi ^3H -thymidine was added to each well; cultures were collected 16 h later on glass-fibre filters using a semi-automatic sample harvester. ^3H -Tdr incorporation assessed by liquid scintillation counting.

Table 1 *M. leprae*-specific and cross-reactive TLC recognize different determinants on a 36K protein purified by a *M. leprae*-specific monoclonal antibody

Antigen	T cell: Antigen specificity:	1G5	1E4	2F9 LEP- VAC- LMU (b)	3B4 Partly cross- reactive (c)	1F9 Completely cross- reactive (d)	3E8 Completely cross- reactive (d)	TLB1	TLB2
		LEP (a)*	LEP (a)						
<i>M. leprae</i>		14.2 ± 4.3	57.9 ± 0.1	88.4 ± 6.2	42.2 ± 2.1	10.0 ± 2.0	70.5 ± 3.5	53.7 ± 5.0	46.7 ± 0.9
36K <i>M. leprae</i> protein		0.1 ± 0.0	24.0 ± 5.5	26.8 ± 0.5	21.4 ± 0.6	0.2 ± 0.0	20.0 ± 0.2	18.2 ± 4.9	17.2 ± 3.4
12K <i>M. leprae</i> protein		0.1 ± 0.1	0.1 ± 0.0	48.8 ± 5.4	0.2 ± 0.1	0.1 ± 0.0	5.9 ± 1.4	0.2 ± 0.0	3.9 ± 0.9
Medium		0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	2.3 ± 1.8	0.1 ± 0.1	0.3 ± 0.0

The results are the mean c.p.m. ($\times 10^{-3}$) $\pm$ s.d. of duplicate cultures. Positive cultures are boxed and are defined as exceeding the mean background proliferation by at least $3 \times$ s.d. and in addition giving $>10^3$ c.p.m. Proliferation assays were carried out as described in Fig. 1 legend. *M. leprae* antigen is Dharmendra lepromin. The 36K protein was purified by the fast protein liquid chromatography system (FPLC; Pharmacia, Sweden) (P.R.K. *et al.*, manuscript in preparation). Briefly, *M. leprae* sonicate was applied to an anion-exchange column (Mono Q, HR5/5; Pharmacia) and eluted with a linear salt gradient. Further purification was carried out on a gel permeation column (TSK G3000 SW; LKB, Sweden). The purity of the antigen was analysed by SDS-polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis immunoperoxidase assay²¹. The 12K protein was purified by affinity chromatography using monoclonal antibody ML06A1 coupled to cyanogen bromide-activated Sepharose 4B, followed by elution with 3 M NaSCN⁹. The optimal stimulatory concentration for the 36K protein was $16 \mu\text{g ml}^{-1}$, and that for the 12K protein, $0.4 \mu\text{g ml}^{-1}$. TLB1 and TLB2 were generated as described in ref. 15.

* For abbreviations see Fig. 1 legend.

of APC, which drastically reduces the number of autologous peripheral blood mononuclear cells needed for the cloning and propagation of antigen-specific T cells.

Twenty-three *M. leprae*-reactive proliferative TLC were selected at random and studied in more detail with regard to their antigen specificity. All 23 TLC were of the T3⁺ T4⁺ T8⁻ phenotype, and were strongly positive for HLA-DR antigens. Twenty-two TLC were restricted in their response by HLA-DR determinants and one was restricted by a hitherto unknown polymorphic HLA-DQ determinant, as assessed by panel and inhibition studies (T.H.M.O. *et al.*, manuscript in preparation).

Two *M. leprae* preparations of different origin, ultrasonicates from 19 other mycobacteria, and the purified protein derivative (PPD) of *Mycobacterium tuberculosis* were presented to these TLC via autologous or HLA (histocompatibility locus antigen) class II-compatible APC. The results (Fig. 1) reveal four patterns of antigen reactivity: (1) TLC recognizing determinants expressed exclusively by *M. leprae* ($n=6$); (2) TLC activated by determinants present on *M. leprae* and cross-reactive with one or two other mycobacterial strains, mainly *Mycobacterium vaccae* and *Mycobacterium lepraemurium* ($n=5$); (3) TLC reactive with several but not all mycobacteria ($n=2$); and (4) TLC triggered by cross-reactive determinants present on all mycobacteria, sometimes with the exception of *Mycobacterium non-chromogenicum* ($n=10$). None of the TLC showed a proliferative response in the presence of APC and either tetanus toxoid or *Candida albicans*, which represented non-mycobacterial control antigens. Similar data were obtained with TLC from two other patients (data not shown).

Recently, five *M. leprae* proteins have been described that contain *M. leprae*-specific antigens recognized by murine monoclonal antibodies^{7-9,14}. The M_r s of these proteins are 12K, 18K, 28K, 36K and (55-)-65K. To investigate whether these antibody-defined *M. leprae* proteins are also recognized by human T cells, we tested the 12K and 36K *M. leprae* proteins which had been purified by using, respectively, the *M. leprae*-specific monoclonal antibodies MLO6A1 (refs 8, 9) and F47-9-1 (refs 8, 10). Six different *M. leprae*-reactive TLC, and a *M. leprae*-generated polyclonal T-lymphoblast culture (TLB)¹⁵ from the same patient (TLB1) and from a second tuberculous leprosy patient (TLB2) were tested.

The data presented in Table 1 show that the *M. leprae*-specific 36K protein is recognized by four of the six TLC tested and by both TLB1 and TLB2, whereas the *M. leprae*-specific 12K purified protein is recognized by only one TLC and (weakly) by one TLB, each of which is also reactive with the 36K protein. As all four TLC reacting with the 36K protein display different antigen-specificity patterns (Fig. 1), they must recognize four different antigenic determinants on the 36K protein, only one of which is clearly *M. leprae*-specific. The finding that only one of the two *M. leprae*-specific TLC (1E4, see Table 1) recognized the 36K protein suggests that the other *M. leprae*-specific TLC tested (1G5) reacts with another *M. leprae*-specific determinant not expressed on the 36K protein. In addition, as only one of the two completely cross-reactive TLC (3E8) tested reacts with the 36K protein, at least two common mycobacterial determinants are defined by these TLC, one of which resides on the 36K protein.

The six TLC and the two TLB have so far failed to respond to 3,6-di-O-methylglucose-(CH₂)₈-bovine serum albumin (BSA), when tested at a wide range of concentrations (5×10^{-3} - $2 \times 10^3 \mu\text{g ml}^{-1}$). This synthetic antigen contains a *M. leprae*-specific monosaccharide component which forms the terminal part of the trisaccharide complex on *M. leprae* phenolic glycolipid. This glycolipid has been shown to trigger *M. leprae*-specific suppressor T cells in lepromatous but not in tuberculous patients, as measured by inhibition of concanavalin A-stimulated peripheral blood mononuclear cell cultures¹³.

Thus, 6 out of 23 human helper TLC recognized at least two separate determinants that are uniquely expressed by *M. leprae*, but which are distinct from those defined by murine monoclonal antibodies, only a few of which have been found to recognize *M. leprae*-specific protein antigens^{7-10,14}. So far, all murine *M. leprae*-reactive TLC cross-react with other mycobacteria^{16,17}. The finding that 3 out of 5 nearly *M. leprae*-specific human TLC also react with *M. vaccae* is in accord with the results of *in vivo* skin tests which revealed a selective cross-reactivity between *M. leprae* and *M. vaccae*¹⁸.

The TLC described here define antigenic determinants on purified *M. leprae* proteins which are capable of triggering T cells. It remains to be elucidated which *M. leprae* antigenic determinants recognized by these helper TLC are involved in

protective immunity as opposed to immunopathology. A comparison of the antigenic determinants defined by TLC from healthy contacts with those defined by TLC from tuberculous leprosy patients may be helpful in distinguishing between the two groups of *M. leprae* antigens. In addition to these two groups, a third group of antigens may be involved in the induction of *M. leprae*-specific suppression^{6,11}. Recently, we have cloned *M. leprae*-reactive suppressor T cells. Thus, we will be able to study whether different antigenic determinants are recognized by cloned helper and suppressor T cells.

Thus the use of TLC and purified *M. leprae* proteins will enable us to define the *M. leprae* antigens involved in T-cell-mediated protective immunity, immunopathology and possibly suppression; these, therefore, are exquisite tools for the rational design of a *M. leprae* vaccine and skin-test reagents^{1,6}.

The T-cell responses against these proteins cannot be inhibited by the relevant monoclonal antibodies (data not shown); this was not unexpected because, as a rule, antibodies and T cells react with different epitopes of the same antigen (see, for example, ref. 19). It is interesting that four out of the six TLC and both TLB react with the 36K protein, whereas only one of these TLC and one TLB respond to one of the four other *M. leprae*-specific proteins known—that is, the 12K protein. This result suggests that T_H cells preferentially recognize immunodominant antigenic determinants²⁰ expressed on the 36K protein. Recently, the genes for the major proteins of *M. leprae* have been cloned and expressed⁷ and indeed the five above-mentioned *M. leprae*-specific proteins have been formed. Therefore, it should now be possible to determine whether one or more of the antigenic determinants on the 36K protein defined by these TLC is formed—in part—by lipid or carbohydrate components.

We thank Dr R. J. W. Rees and the late Dr C. C. Shepard for providing us with *M. leprae* antigens; Dr D. L. Leiker for his help in obtaining blood samples from leprosy patients; Arend Kolk and Frits Koning for monoclonal antibodies; Teunis Eggelte for synthetic saccharide antigens; John Haanen and Earl Johanns for technical assistance; and Tiny van Westerop for drawing part of the figure and preparing the manuscript. This study was supported in part by the Foundation for Medical Research (FUNGO grant 13-83-01), the Immunology of Leprosy (IMMLEP) component of the UNDP/WORLD Bank/WHO Special Programme for Research and Training in Tropical Diseases, and the Netherlands Leprosy Relief Association (NSL). *Note added in proof:* Recent experiments show that analysis of a recombinant 36K *M. leprae*-specific protein, expressed in *Escherichia coli*, reveals that different *M. leprae*-specific protein determinants are detected by these *M. leprae*-specific TLC.

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Identification of a secretory granule-binding protein as caldesmon

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Stimulation of adrenal chromaffin cells results in a rise in the concentration of intracellular free calcium^{1–3} which initiates catecholamine secretion by exocytosis^{4,5}. An understanding of the molecular basis of exocytosis will require knowledge of the sites of action of calcium. A role for calmodulin has been implicated in secretion from chromaffin cells^{6,7}, and isolated granule membranes bind both calmodulin⁸ and a series of cytosolic proteins^{9–12} in a calcium-dependent fashion. Here, we demonstrate that one of the cytosolic granule-binding proteins with a relative molecular mass (*M_r*) of 70,000 (70K) is a form of the calmodulin-regulated actin-binding protein caldesmon, first isolated from smooth muscle¹³. Cytoplasmic gels assembled from an adrenal medullary extract in the absence of Ca²⁺ contained actin and the 70K protein. The association of both of these proteins with the cytoplasmic gel was inhibited by a micromolar concentration of Ca²⁺. In addition, we have demonstrated that the 70K protein is localized at the periphery of chromaffin cells. These results are consistent with the notion that 70K protein (caldesmon) has a role in regulating the organization of actin filaments of the cell periphery during the secretory process.

A number of bovine medullary cytosolic proteins bind to chromaffin granule membranes with a range of affinities for Ca²⁺ (refs 9–12). Among those that bind with the highest affinity (Fig. 1a, b) is a polypeptide with an approximate *M_r* of 70,000. This major granule-binding protein was used as an immunogen in the preparation of specific antisera (Fig. 1). Anti-70K antiserum recognized the 70K polypeptide on immunoblots of granule-binding proteins (Fig. 1c) and adrenal medulla homogenates. In immunoblots of homogenates of a variety of rat tissues, anti-70K antiserum recognized a 70K polypeptide in brain, pancreas, heart and liver but not in skeletal muscle (Fig. 1f–i). An additional 130K polypeptide was recognized by anti-70K antiserum in brain and liver. A non-immune mouse antiserum did not recognize any polypeptides on immunoblotting. The 70K polypeptide in the adrenal homogenate (Fig. 1d, e) and the granule-binding protein fraction, and the 70K and 130K polypeptides recognized by anti-70K in brain homogenates, were found to be heat-stable.

The protein caldesmon was first isolated from chicken gizzard as a 150K calmodulin-binding F-actin regulatory protein¹³. Subsequently, caldesmon was shown to be present in a range of tissues and cell types^{14,15} but absent from skeletal muscle¹⁴. Caldesmon is now known to exist in a 120–140K form and a 71–77K form; both forms bind to calmodulin and F-actin and are heat-stable^{15,16}. There is a remarkable similarity between these caldesmon forms and the polypeptides recognized here by anti-70K, including the ability of 70K from the granule-binding preparation to bind to calmodulin¹¹. That the 70K and 130K proteins are forms of caldesmon was confirmed by the finding that anti-70K recognized the purified turkey gizzard 140K caldesmon on immunoblotting (Fig. 2a, b).

Extracts from adrenal medulla incubated at 25 °C in the absence of Ca²⁺ form supramolecular cytoplasmic gels. Using peptide mapping in comparisons with skeletal muscle proteins, we have shown that actin and myosin are major constituents of these gels (our unpublished observations). Gels assembled from extracts containing >1 μM free Ca²⁺ were depleted of both actin and a 70K polypeptide (Fig. 2c–i). Immunoblotting with

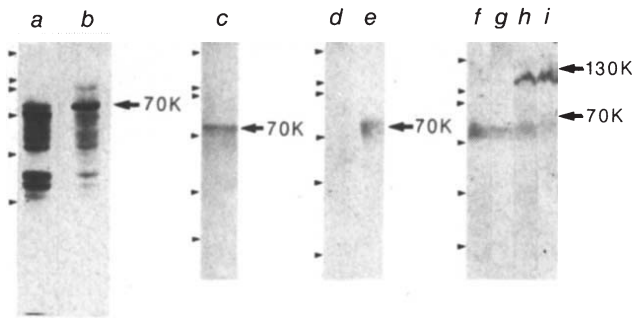


Fig. 1 Characterization of anti-70K antiserum. Granule-binding proteins were prepared⁹ by binding from bovine medullary cytosol in the presence of 1 mM Ca^{2+} and elution at 100 μM free Ca^{2+} (a), followed by elution with EGTA (b). c–i, Immunoblotting with anti-70K antiserum of: granule-binding protein fraction, eluted with EGTA (c), and the heat-precipitated (d) and heat-stable (e) fractions of bovine adrenal medullary homogenate; and homogenates of rat heart (f), pancreas (g), liver (h) and brain (i). Differences in the apparent migration of the 70K polypeptide between gels are due to differences in electrophoretic conditions. In each case the positions of M_r markers are indicated by arrowheads on the left (M_r , from the top: 205K, 116K, 94K, 67K, 43K and 29K). **Methods.** Anti-70K antiserum was prepared by excising gel bands from tracks of granule-binding proteins as in b. The gel slices were homogenized in phosphate-buffered saline (PBS) and injected into mice with Freund's complete adjuvant three times, with intervals of 14 days between injections. The mice were killed and bled 14 days after the last injection and a γ -globulin fraction prepared from the serum by sodium sulphate precipitation. Antisera produced in two different mice gave essentially identical results. For immunoblotting, proteins were separated on 10 or 12.5% polyacrylamide slab gels and transferred to nitrocellulose sheets. After staining the proteins with Ponceau S, the nitrocellulose was washed in PBS, incubated in 3% bovine serum albumin (BSA), 0.2% Triton X-100 in PBS for 10 min, then incubated overnight at 4°C with anti-70K (1:50 dilution). Bound antibody was detected by incubation with anti-mouse biotin (diluted 1:50; Amersham) for 60 min, followed by incubation with horseradish peroxidase (HRP)-streptavidin (diluted 1:150; Amersham) for 30 min and development with diaminobenzidine. All antibodies were diluted in 3% BSA, 0.2% Triton X-100 in PBS. Homogenates were prepared by homogenization in 5 mM Tris-HCl, 4 mM EGTA, pH 8.0 and 180 μg protein was electrophoresed per track. Adrenal medullary homogenate was boiled for 3 min before separation, by centrifugation, into heat-precipitable and heat-stable fractions.

anti-70K demonstrated that the antiserum recognized the Ca^{2+} -regulated 70K polypeptide of the gel as well as a minor 130K polypeptide (Fig. 2k, l); this provides additional confirmation that the 70K protein is the actin-binding protein caldesmon and demonstrates that the assembly of actin and 70K into supramolecular gels is inhibited by micromolar Ca^{2+} concentrations.

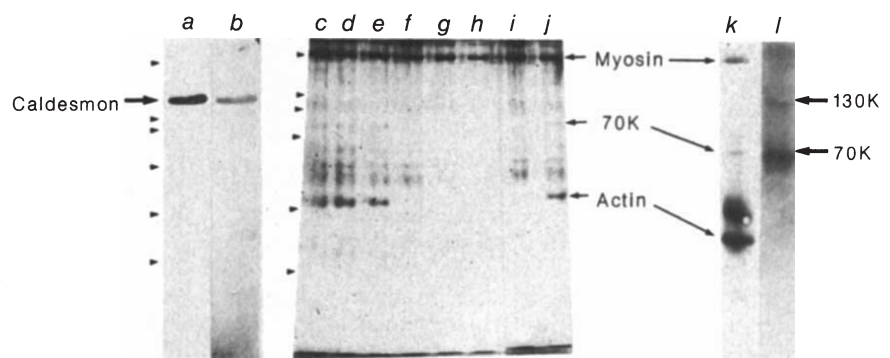
The localization of 70K in bovine adrenal chromaffin cells was determined by immunoperoxidase staining of bovine chromaffin cells in primary culture (Fig. 3). The cultures also contained flat non-chromaffin cells¹⁷ which showed weak, diffuse staining with anti-70K. In chromaffin cells, anti-70K antiserum produced intense staining of regions at the cell periphery. We have also found that on cryostat sections anti-70K stains the periphery of rat adrenal chromaffin cells as well as cells from a wide range of other tissues (R.D.B. and K.-M.N., unpublished observations). Anti-70K stained stress fibres in fibroblasts in culture^{15,18} and gave a pattern of staining in small intestine that was indistinguishable from that described for antibody to gizzard caldesmon¹⁴. The localization of 70K in chromaffin cells resembles that of α -fodrin¹⁹, which suggests that these two proteins may form part of a subplasmalemmal cytoskeleton. However, α -fodrin was localized as a continuous ring around the cell periphery in control cells and as patches following stimulation, whereas 70K was already localized along patches of the cell periphery in control cells (Fig. 3). We detected no effect of stimulation on the localization of 70K in chromaffin cells.

Thus, we have demonstrated that a protein isolated on the basis of its ability to bind reversibly to chromaffin granule membranes in the presence of Ca^{2+} is a form of the actin-binding protein caldesmon. Furthermore, caldesmon and actin assemble from adrenal medullary extracts into supramolecular gels only when the free Ca^{2+} concentration is $<1 \mu\text{M}$. It has been shown previously that, in the absence of Ca^{2+} , caldesmon stimulates actin assembly²⁰, crosslinks F-actin¹⁶ and inhibits the regulation of F-actin gelation by filamin^{21,22}. All these functions of caldesmon are inhibited by Ca^{2+} in the presence of calmodulin, to which caldesmon then binds preferentially^{13,16,20,22}. In the adrenal chromaffin cell, caldesmon may be expected to control a reorganization of F-actin at the cell periphery, including solation of the subplasmalemmal actin network following the rise in intracellular free Ca^{2+} concentration during stimulation.

A role for changes of cytoskeletal organization in secretion from chromaffin cells has previously been suggested from the observations that chromaffin granules bind F-actin²³ and increase the viscosity of F-actin in a Ca^{2+} -inhibitable manner²⁴

Fig. 2 Recognition by anti-70K antiserum of turkey gizzard caldesmon and a Ca^{2+} -regulated protein in medullary cytoplasmic gels. Turkey gizzard 140K caldesmon was transferred to nitrocellulose and revealed by Ponceau S staining (a) and immunoperoxidase staining with anti-70K (b). Cytoplasmic gels assembled from medullary extracts in the presence of 0.1 (c), 0.5 (d), 1.0 (e), 2.5 (f), 5.0 (g), 7.5 (h) or 10 μM (i) free Ca^{2+} or EGTA (j) are shown on a Coomassie blue-stained gel, and in the presence of EGTA after transfer to nitrocellulose and staining by Ponceau S (k), followed by anti-70K (l). The positions of M_r markers are indicated by arrowheads on the left (M_r , from the top: 205K, 116K, 94K, 67K, 43K, 29K).

Methods. Caldesmon was prepared from turkey gizzard as described previously¹⁶. For the preparation of cytoplasmic gels, adrenal medullae were homogenized in 1 mM EDTA, 10 mM mercaptoethanol, 0.5 mM ATP, 40 mM Tris-HCl pH 7.4 and centrifuged at 100,000g for 90 min at 4°C. Aliquots were dialysed against 5 mM EGTA, 10 mM mercaptoethanol, 0.5 mM ATP, 40 mM Tris-HCl pH 7.0, with CaCl_2 added to give a calculated free $[\text{Ca}^{2+}]$ of 0.1–10 μM . Cytoplasmic gels were assembled after addition of MgCl_2 to 8 mM by incubation at 25°C for 60 min, harvested by centrifugation at 13,000g for 7 min at room temperature, and contaminating membranous material was removed by washing with buffer containing 0.5% Triton X-100.



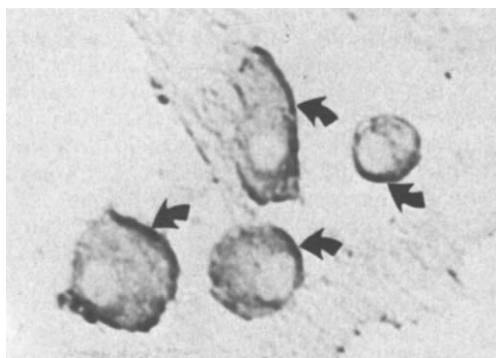


Fig. 3 Immunoperoxidase staining of bovine chromaffin cells in primary culture by anti-70K antiserum. Non-chromaffin cells in the culture¹⁷ were only weakly stained. Chromaffin cells (arrowed) have intense staining around the internal periphery of the cells which in most cases does not form a continuous ring.

Methods. Bovine adrenal chromaffin cells were dissociated and maintained in monolayer culture for 2 days as described previously¹⁷, after which they were fixed with 4% formaldehyde in PBS and air-dried. After washing in PBS, immunoperoxidase staining with anti-70K antiserum was done using essentially the same conditions as those used for immunoblotting (see Fig. 1 legend).

and that stimulation of chromaffin cells results in a redistribution of α -fodrin¹⁹. Of importance for future investigations is the relationship between caldesmon and α -fodrin at the cell periphery during exocytosis. The demonstration of a reversible

association of caldesmon with granule membranes suggests an additional function for caldesmon in regulating the interaction of secretory granules with the cytoskeleton during the secretory process.

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Estimation of the polarity of the protein interior by optical spectroscopy

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Crystallographic studies of myoglobin have shown that the haem pocket is lined with nonpolar amino-acid residues¹. In order to estimate the true polarity of the interior of proteins, certain studies have used bound fluorophores²⁻⁷, the spectroscopic properties of which reflect the polarity of their environment. These studies have most often used 1-amino-8-naphthalene sulphonate (ANS) as a probe, but a more suitable probe, in principle, is 6-propionyl 2-(N,N-dimethyl)aminonaphthalene (PRODAN)⁸. We have synthesized a molecule with the advantageous spectroscopic properties of PRODAN but with a higher affinity for apomyoglobin: 2'-(N,N-dimethyl)amino-6-naphthoyl-4-trans-cyclohexanoic acid (DANCA), and report here its use to determine the polarity of the myoglobin haem pocket. Our results show that the pocket is actually a polar environment, and the polarity can be accounted for by peptide amide dipoles.

Polar chromophores and fluorophores are molecules that exhibit large differences in the electronic distribution of the ground and fluorescent states. The local polarity of a biological system harbouring such molecules can be estimated by comparison of the spectral properties in that environment with those of the probe in solvents of known polar characteristics. The ideal polar fluorescent probe should have no sources of charge besides two readily identifiable monopoles, and the dipole moment that they determine should change strength but not direction in the excited state. Most important, the probe should be soluble in a range of solvents that cover the polarity scale,

from apolar solvents like cyclohexane to the most polar ones like alcohol or water. PRODAN is a fluorophore probe which approaches this standard (Fig. 1); its synthesis and spectral properties have been described elsewhere⁸. The positive monopole resides at the amino nitrogen and the negative one at the keto oxygen. The dipole moment in the ground state is ~2 D and that of the fluorescent state 18-20 D. In cyclohexane the absorption and emission are centred at 341 and 401 nm respectively, in water they are located at 364 and 531 nm. The absorption and emission bands in water are much broader than in cyclohexane.

Attempts to observe the changes in spectra that result when PRODAN is bound to apomyoglobin are hampered by the relatively low affinity of this molecule for this binding site. To improve the binding affinity while keeping the spectral characteristics of PRODAN intact, we synthesized DANCA (Fig. 1 and G.W. and F. J. Farris, unpublished data). The negatively charged carboxyl was introduced to improve the affinity for the apoprotein, following the example of haem itself, while the cyclohexane ring was intended to hold the carboxyl group away from the spectrally relevant aminoaphthoyl moiety. This isolation reveals itself in the spectral identity of PRODAN and DANCA in media in which their common solubility permits comparison.

At 24 °C apomyoglobin binds one DANCA molecule with $K_D = 1.2 \times 10^{-5}$ M. Bound DANCA is stoichiometrically displaced by the addition of one equivalent of haem, indicating a unique location in the haem pocket of myoglobin³. The absorption spectrum of the protein-dye complex (Fig. 1) is centred at 362 nm (27,620 cm⁻¹) and has a halfwidth of 2,790 cm⁻¹. Cooling the sample to 4 °C shifts the absorption to 366 nm (27,320 cm⁻¹) and causes it to narrow to 2,480 cm⁻¹. The spectrum of DANCA in buffer is centred at 360 nm (27,780 cm⁻¹), with a halfwidth of ~3,600 cm⁻¹ and no appreciable temperature dependence. In cyclohexane the absorption of PRODAN is centred at 341 nm (29,326 cm⁻¹) and has a halfwidth of 2,100 cm⁻¹. The shift in absorption with respect to cyclohexane is direct evidence of the considerable polarity of the haem pocket. The temperature dependence of the absorption indicates

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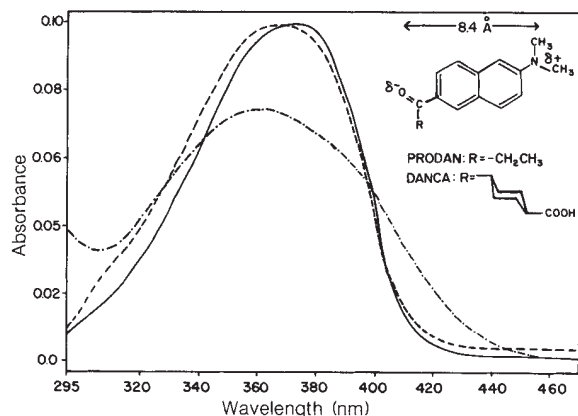


Fig. 1 The absorption spectra of a solution containing 200 μM apomyoglobin and 5.7 μM DANCA (95% bound) at 4 $^{\circ}\text{C}$ (—) and at 24 $^{\circ}\text{C}$ (---). Also shown is the absorption spectrum of a 5.7- μM solution of DANCA in buffer at 24 $^{\circ}\text{C}$ (-.-.-). Inset: structure of PRODAN and DANCA. DANCA was synthesized in this laboratory; purity was verified using HPLC. The concentration of DANCA in water was determined spectrophotometrically using $E = 14,000 \text{ cm}^2 \text{ mmol}^{-1}$ at 360 nm. Haemin and sperm whale myoglobin were purchased from Sigma. Apomyoglobin was prepared according to the method of Teale¹³. All experiments involving protein were carried out in 20 mM bis-Tris buffer, pH 6.0. Before an experiment, the apomyoglobin was centrifuged at 20,000 g at 4 $^{\circ}\text{C}$ for 40 min to remove aggregates. The concentration of apomyoglobin was determined from the absorbance at 280 nm using $E = 13,500 \text{ cm}^2 \text{ mmol}^{-1}$ (ref. 14).

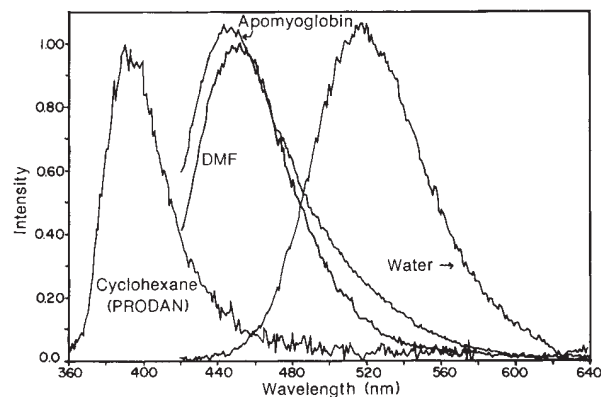


Fig. 2 The fluorescence of DANCA bound to apomyoglobin and in three solvents of different polarity.

that the population becomes more heterogeneous with increasing temperature. This can result from small changes in the binding site or from the appearance of new fluorophore orientations in an unchanged binding site. Regardless of origin, it is clear that the bound dye has, at either temperature, a much narrower distribution of interactions than in the aqueous buffer.

The fluorescence of DANCA in pH 6.0, 20 mM bis-Tris buffer at room temperature has an average wavenumber of $18,826 \text{ cm}^{-1}$ (531 nm). On titration with apomyoglobin, this fluorescence is replaced by a new one centred at $21,755 \text{ cm}^{-1}$ (459 nm) (Fig. 2) and the intensity increases by a factor of four. This emission spectrum is almost identical to those of PRODAN or DANCA in *N,N*-dimethylformamide (DMF) at room temperature⁸ or in a glycerol at -40°C ^{9,10}; it is also strongly redshifted with respect to the fluorescence spectra in nonpolar media like cyclohexane (401 nm), or weakly polar ones like chloroform (440 nm) or even acetone (452 nm).

The heterogeneity of the probe interaction is revealed by the dependence of the fluorescence spectrum on the excitation wavelength and the changes in interaction in the excited state are revealed by its temperature dependence^{9,10}. Figure 3⁹ shows the average fluorescence energy and bandwidth of PRODAN in glycerol as a function of excitation wavelength at -42°C and at 1°C . At the lower temperature, glycerol is a vitrified solid and its interactions with the dye at emission are determined by the ground state charge distribution about the fluorophore. In this condition, excitation at the longer wavelengths selects fluorophore populations possessing more favourable energies of interaction with solvent and therefore results in progressively redder emissions; the fluorescence excited at the red edge of the absorption shows the largest shift and the smallest bandwidth. At the higher temperature, 1°C , the rate of solvent dipole reorientation exceeds the rate of emission and causes the fluorescence spectrum to become almost independent of the excitation wavelength. The emission of DANCA-apomyoglobin (Table 1) also shows dependence on excitation wavelength but, in contrast to the data in Fig. 3, the spectral changes are not diminished by raising the temperature within the range in which the protein remains in its native conformation.

The dependence of the fluorescence spectrum on the exciting wavelength shows that we are dealing with a heterogeneous population of DANCA-apomyoglobin complexes, although occupation is restricted to a single molecule. This result is not surprising as haem itself binds in two orientations within its pocket¹¹.

At 24 $^{\circ}\text{C}$, the absorption of DANCA-apomyoglobin is shifted $-1,706 \text{ cm}^{-1}$ and the emission $-2,751 \text{ cm}^{-1}$ with respect to the corresponding values of PRODAN in cyclohexane. In the complete absence of relaxation of the polar surroundings, we expect equal displacements of the absorption and emission with respect to those observed in an apolar solvent like cyclohexane. The observed difference discloses the existence of a relaxation that increases the Stokes shift expected in a rigid medium by about $-1,000 \text{ cm}^{-1}$ and is substantially complete at 4 $^{\circ}\text{C}$. Its course at lower temperatures was explored by observations of the fluorescence of DANCA-apomyoglobin in 60% glycerol-aqueous buffer. We found that the characteristic spectrum of DANCA-apomyoglobin at room temperature is not disturbed by addition

Table 1 Dependence of the fluorescence of DANCA bound to apomyoglobin on excitation wavelength

Excitation (nm)	Low temperature*		High temperature*	
	$\Delta\bar{\nu}_g$	Halfwidth	$\Delta\bar{\nu}_g$	Halfwidth
359	21,818	1,652	21,755	1,707
427	21,119	1,552	20,985	1,636
Difference	-699	-100	-770	-71

All energies are given in wavenumbers (cm^{-1}).

* At 359 nm, low temperature is 4 $^{\circ}\text{C}$ and high temperature is 24 $^{\circ}\text{C}$; on excitation at 427 nm, low temperature is 6 $^{\circ}\text{C}$ and high temperature is 22 $^{\circ}\text{C}$.

of glycerol. At -34°C the spectral relaxation observed in buffer at higher temperatures is reduced to -200 cm^{-1} and at -74°C it has disappeared altogether.

We draw two conclusions from our observations. First, the protein provides a polar environment that induces a shift in the average absorption of $\sim -1,700 \text{ cm}^{-1}$ with respect to the unperturbed absorption of the probe; this environment is therefore quantitatively similar to that observed in solutions of PRODAN or DANCA in DMF. At -74°C the fluorescence is similarly displaced. Second, a relaxation process takes place during the excited state and results in an additional redshift of the fluorescence of $\sim 1,000 \text{ cm}^{-1}$. It is already incipient at -35°C and virtually complete at 4 $^{\circ}\text{C}$.

The origin of the relaxation is obscure; however, it seems clear that the time-independent effects on the absorption, and the emission of the probe at the lowest temperature can be

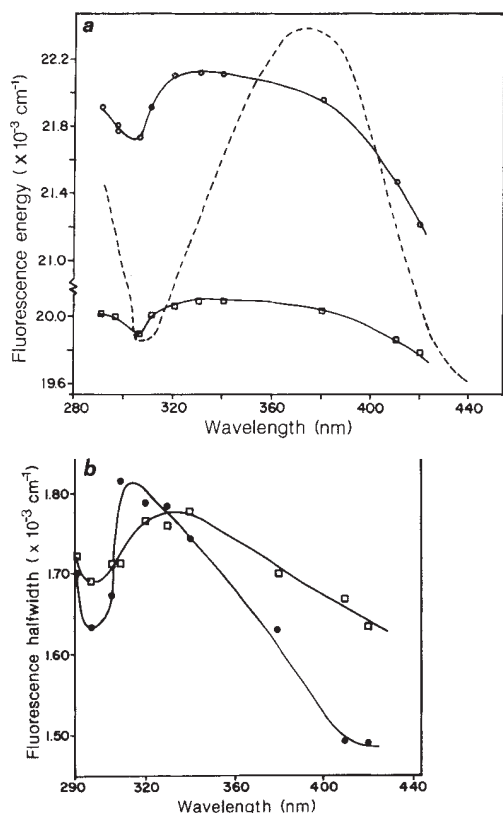


Fig. 3 The excitation wavelength dependence of the emission of PRODAN in glycerol at two temperatures. *a*, Average energy of the fluorescence against excitation wavelength at -42°C ($\circ$) and 1°C ($\square$). The fluorescence excitation spectrum of PRODAN in glycerol at -42°C is given by the broken line. *b*, Fluorescence bandwidth against excitation wavelength at -42°C ($\bullet$) and 1°C ($\square$). All fluorescence spectra were recorded on a modified version of the single-photon counting spectrofluorimeter described by Jameson *et al.*¹⁵, and were corrected for monochromator transmission and photomultiplier tube response. The fluorescence spectra of the DANCA-apomyoglobin complex were deconvolved from the total spectra, which included contributions from free DANCA, by using a nonlinear least-squares fitting procedure¹⁶.

explained in terms of discrete electrostatic interactions between the probe and protein and that the strength of these interactions corresponds to a medium of considerable polarity. This result is at variance with previous conclusions on the polarity of the haem pocket based on the ANS-apomyoglobin complex³. We note that the virtual insolubility of ANS in truly nonpolar solvents excludes the possibility of establishing a reliable polarity scale for this probe. Additionally, the absorption spectrum and the effect of temperature on the absorption and emission were not evaluated.

Crystallographic studies of myoglobin have shown that the haem pocket is lined with nonpolar amino-acid residues¹, yet our results indicate that the binding site is strongly polar. This inconsistency is only apparent; the fluorophore monopoles can readily interact with the protein amide dipoles through the low dielectric medium provided by the nonpolar amino acids. As a direct test of this supposition, we have calculated the electrostatic interaction energy of DANCA with the partial charges at each of the nitrogen and oxygen atoms of the peptide backbone. In this calculation it was assumed that the probe molecule is located in the plane defined by the haem in the crystallographic structure of deoxymyoglobin and that the coordinates of the other atoms in the molecule are unchanged when the haem is substituted by the probe molecule. Figure 4 shows a contour map of calculated interaction energies of the peptide bond

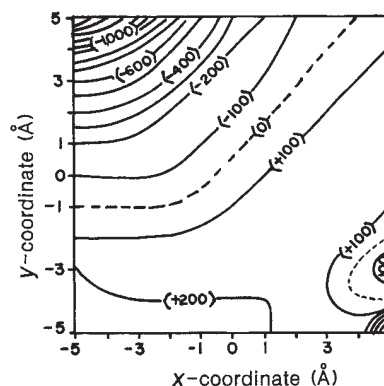


Fig. 4 Map of the electrostatic interaction energy of the peptide bond dipoles with a partial charge located in the plane occupied by the haem in the crystallographic structure of deoxymyoglobin. The partial charge equals the charge of the positive monopole of DANCA in the excited electronic state ($\sim -0.56\text{ e}$) minus its charge in the ground state ($\sim -0.06\text{ e}$); the strength of the DANCA monopoles follows from the known dipole moments in ground and excited state, 2 and 18 D, respectively, and the distance between the monopoles, 8.4 Å. The x-coordinate is the direction defined by a line drawn from Fe^{2+} and N-1 of the porphyrin ring, the y-coordinate is along the line between Fe^{2+} and N-2, and the z-coordinate is perpendicular to the ring. The energies are given in units of cm^{-1} to facilitate comparison with the spectroscopic results. Contour lines represent changes of 100 cm^{-1} in the interaction energy. Energies were calculated assuming a dielectric constant of 3 for the interior of the protein, and a dipole moment of 3.7 D for the peptide bond.

charges with a positive monopole successively placed at the nodes of an $11 \times 11\text{-Å}$ grid situated in the haem plane. A dielectric constant of 3 was assumed for the intervening medium. The contour lines show calculated differences between the electrostatic interaction energies of all the protein peptide dipoles with the amino nitrogen of DANCA, in both the ground and excited states, expressed in wavenumbers to permit direct comparison with the spectral shifts. The values for the negative monopole (DANCA keto oxygen) are equal in magnitude but with opposite sign. For each position that can be chosen as a location for the amino nitrogen of the probe, the total change in interaction energy on excitation equals the energy shown at that point plus the negative of the energy at a point placed 8.4 Å away. Several probe orientations yield spectral displacements well in excess of $-1,000\text{ cm}^{-1}$. These values are close to the difference between the absorption of DANCA-apomyoglobin at room temperature or that of the emission at -74°C , and the respective values for PRODAN in cyclohexane. However, we do not wish to stress the coincidence of observation and calculation; no exact calculation could be made without knowing the atomic structure of the DANCA-apomyoglobin complex. Our intention here is to demonstrate qualitatively the contribution of the peptide bonds to the polarity of the interior of proteins rather than to analyse particular properties of myoglobin.

Warshel *et al.*¹², in re-examining the theories concerning the interactions of charges located within proteins, point out that the similarity of the dissociation constants of proton-bearing groups within the protein and in water force the conclusion that the protein is polar in their immediate vicinity, and that previous theoretical treatments had neglected the role of peptide bonds in the stabilization of charges. Our results corroborate these considerations by demonstrating that a large portion of the polarity registered by a spectroscopic probe placed in the haem pocket of myoglobin must be attributed to the peptide dipoles.

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Human N-myc is closely related in organization and nucleotide sequence to c-myc

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N-myc, a cellular gene related to the **c-myc** proto-oncogene¹⁻⁴, was originally identified on the basis of its very frequent amplification and overexpression in a restricted set of tumours, most notably human neuroblastomas^{1,2}. That **N-myc** may have a causal role in the genesis of these tumours is suggested by the observation that in the rat embryo fibroblast co-transformation assay^{5,6} it has a transforming potential similar to that of **c-myc**^{7,8}. The apparent structural and functional homology of **N-myc** and **c-myc** suggests that they may be members of the same proto-oncogene family. However, despite these apparent similarities, expression of the two genes appears to be dramatically different with respect to tumour specificity, as well as tissue and developmental stage specificity^{9,10}. To further elucidate the common and unique aspects of **N-myc** and **c-myc** gene structure and function in normal and transformed cells, we have determined the organization of human **N-myc** and the nucleotide sequence of its messenger product, and we report here that **N-myc** and **c-myc** have a similar intron/exon structure and that their protein products share regions of significant homology.

To isolate an **N-myc**-specific complementary DNA clone, we prepared a λ phage Charon 16A cDNA library from total poly(A)⁺ RNA of the IMR-32 human neuroblastoma cell line and screened the library for hybridization to various **N-myc** genomic probes. Initial screenings with the **N-myc** genomic probe NB-19-21 which derives from the more 5' region of the gene (see Fig. 1a) yielded no clones. Thus, we isolated a genomic clone containing regions of the gene both 5' and 3' to NB-19-21 (Fig. 1a), identified restriction fragments from the 3' portions which contained coding sequences but not repetitive sequences, and used these to further screen the library. This approach yielded multiple **N-myc**-specific cDNA clones, the longest of which was ~1 kilobase (kb) (pI1-1; Fig. 1b). To obtain longer cDNA sequences we hybridized a primer prepared from pI1-1 (Fig. 1b) to mRNA from the human neuroblastoma cell line NB-16, extended the primer with reverse transcriptase, prepared a Charon 16A library of the primer-extended cDNA, and

screened the resulting library with NB-19-21 (see Fig. 1 legend). In this way we isolated an additional cDNA clone, p9D (Fig. 1b), which when combined with pI1-1 represents 1.7 kb of unique sequence.

Attempts to extend primers isolated from the 5' end of clone 9D were unsuccessful due to a strong stop in the reverse transcription reaction; various analyses indicated that this strong stop resulted from extensive secondary structure in this region of the sequence (see Fig. 2). To bridge this strong stop, we used S₁ nuclease mapping (fragments 7,7' and 8; see Fig. 1c) to identify coding sequences in the corresponding region of the genomic clone. A primer isolated from these coding sequences (a 500-base pair (bp) *XhoI*-*Bam*HI fragment from NB-19-21; Fig. 1a) was hybridized to NB-16 mRNA, extended and cloned. Screening the library with a 1-kb *Bam*HI-*Eco*RI genomic fragment shown by S₁ nuclease analysis to contain **N-myc** coding sequences (BR-1; Fig. 1a) yielded several clones, the longest of ~1 kb (p0; Fig. 1b). Colinearity of these cDNA sequences with the mRNA was confirmed by S₁ nuclease protection experiments (probes 1-5; Fig. 1c). Together, the cDNA clones and the genomic fragment bridging them represent 2.7 kb of unique sequence, almost enough to encode the entire **N-myc** message (~3.0 kb). Additional transcribed sequences lie upstream of the 5' end of the cDNA clone (see below).

Comparison of the nucleic acid sequence of the **N-myc** cDNA and genomic clones, coupled with various S₁ nuclease mapping experiments, indicated that **N-myc** has three exons; in analogy to **c-myc** gene, we refer to these exons as 5'-exon 1-exon 2-exon 3-3' (Fig. 1a). Figure 2 shows the nucleotide sequences of the **N-myc** cDNA clones and of selected regions of the genomic clone containing sequences complementary to **N-myc** mRNA (as defined by S₁ protection). The presence of a poly(A) tail and an adenylation signal confirms the orientation of the genomic and cDNA clones and defines approximately the 3' terminus of the **N-myc** gene (Figs 1a, b, 2).

Restriction endonuclease mapping and Southern blot analyses (data not shown) indicate that the 5' portion of the 9D cDNA clone spans a discontinuous region in the genomic clone. Comparison of the nucleotide sequence of this region of the cDNA clone with the relevant regions of the **N-myc** genomic clone confirms that sequences contiguous in cDNA are separated by an intron of ~2,500 bp in the genomic clone (Figs 1a, b, 2). That this discontinuity was not a cloning artefact was confirmed by appropriate S₁ nuclease protection experiments (using fragment 8 in Fig. 1c). The sequences CAG/GTAAAG, spanning the 3' boundary of exon 2, and TCTTCTCTCAG/AT, spanning the 5' boundary of exon 3, are in agreement with the predicted consensus sequences for the donor and acceptor splice junctions¹¹. Extensive restriction endonuclease analyses (not shown) confirm the colinearity of the cDNA and genomic clone within the proposed exon 3 sequence. Thus, exon 3 extends for 1,526 bp between the splice site and the poly(A) tail (Fig. 2). The 5' boundary of exon 2, located by S₁ nuclease protection analyses, lies ~170 bp 5' to the *XhoI* site within the NB-19-21 region (analyses used fragments 7 and 7' of Fig. 1c); the exact boundary (nucleotide 680, Fig. 2) was determined by comparing the nucleotide sequences of the corresponding regions of the genomic clone and cDNA clone p0. Thus, exon 2 extends 902 bp from this upstream boundary to the 3' splice donor site. The sequences TTGCAG/TGT, spanning the 5' boundary of exon 2, and CCG/GTTCGT, spanning the 3' boundary of exon 1, correspond respectively to the acceptor and donor splice junction consensus sequences¹¹. Sequences in the 5' cDNA clone p0 extend 314 bp upstream of the splice donor site at the 3' boundary of exon 1.

Because S₁ nuclease protection experiments (using fragment 6 of Fig. 1c) indicated that additional transcribed sequences lie upstream of the end of this clone, we used primer extension to define the 5' boundary of exon 1. Primer extension of a *NarI* fragment isolated from the 5' end of p0 (nucleotides 309-375;

Fig. 1 Organization of human *N-myc*. **a**, Partial restriction map of the *N-myc* genomic clone NG-2. Boxes are exons; shaded boxes denote untranslated sequences, open boxes translated sequences. The heterogeneity of the 5' terminus of exon 1 is indicated by sawtoothing. Genomic fragments used as probes to screen the cDNA libraries and in Northern blot analyses are indicated at the top. Arrows below the map indicate genomic fragments that have been sequenced. Restriction sites: Ac, *AccI*; Av, *AvaI*; B, *BamHI*; Bg, *BglII*; H, *HincII*; Hf, *HinfI*; P, *PstI*; R, *EcoRI*; S, *SmaI*; X, *XhoI*; Xb, *XbaI* (not all sites are shown). **b**, Partial restriction map of the *N-myc* cDNA. Open box denotes the structure of the *N-myc* cDNA deduced from the cDNA clones, S₁ nuclease mapping of relevant regions of the genomic clone and primer extension. The numbers within the boxes indicate the positions of the three exons. The shaded area within exon 2 represents the region of composite cDNA derived from genomic coding sequences; the stippled area in exon 1 represents the region derived by primer extension and S₁ nuclease mapping. The heterogeneity of the 5' end of the mRNA is indicated by sawtoothing. The cDNA clones are shown beneath the map. The boxed portion of cDNA clones p9D and p0 indicates the position of the primer used in the construction of these clones. Arrows indicate sequencing strategies. **c**, S₁ nuclease mapping of the coding regions of the genomic clone and of the cDNA clones. Fragments used as probes in the S₁ protection experiments are shown. The locations of the protected fragments are indicated by solid boxes; for probes 6 and 8, the location was confirmed by sequence analysis and cDNA cloning. For probe 9, the multiplicity of the protected fragments is indicated by a hatched box. The size of the protected fragment is given in parentheses. An asterisk at the end of a fragment indicates that the probe was terminally labelled; all other probes were uniformly labelled.

Methods. The *N-myc* genomic clone NG-2 was obtained by screening a Charon 35 recombinant phage library (prepared from size-fractionated, *MboI* partially-digested DNA⁴⁴ from the LA-N-5 human neuroblastoma cell line⁴⁵) with the 2-kb *EcoRI* fragment NB-19-21 (ref. 1). cDNA clones p11-1 and p11-3 were isolated from a cDNA library⁴⁶ prepared from total poly(A)⁺ RNA from the IMR-32 cell line⁴⁷ and screened with a 1.6-kb *HpaII-EcoRI* fragment of the genomic clone which was shown to contain coding sequences but not repetitive sequences as follows. *EcoRI* fragments from the genomic clone 3' of NB-19-21 were further digested with various restriction enzymes, the DNA fractionated on agarose gels and transferred to nitrocellulose. Duplicate blots were probed with either nick-translated human DNA (to detect repetitive sequences) or first-strand cDNA synthesized from RNA from the NB-16 human neuroblastoma cell line⁴⁸ (to detect coding sequences). cDNA clone 9D was derived as follows. A terminally labelled, single-stranded *HaeIII* fragment isolated from p11-1 (nucleotides 2,481-2,745 in Fig. 2) was used in a primer extension reaction⁴⁹ with cytoplasmic poly(A)⁺ RNA⁴⁶ from the NB-16 cell line. This DNA/RNA hybrid was converted to double-stranded (ds) DNA⁵⁰ and cloned into the λ phage vector Charon 16A⁴⁶. The library was screened with the NB-19-21 probe. The cDNA clone p0 was obtained by primer extension of a 500-bp *XhoI-BamHI* genomic fragment (nucleotides 852-1,357 in Fig. 2) and cloning of the ds-cDNA as described above. The library was screened with the 1-kb *BamHI-EcoRI* genomic fragment, BR-1. For S₁ nuclease mapping, fragments were subcloned into the M13 phage vector mp8 or mp19 and single-stranded, uniformly labelled DNA probes were prepared as described previously⁵¹. Alternatively, the DNA was terminally labelled after restriction endonuclease digestion and single-stranded probes were isolated from a strand separation gel⁴⁶. Labelled probes were hybridized with 20-250 ng of NB-16 cytoplasmic poly(A)⁺ RNA in 80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 42°C or 50°C overnight, and digested with 6,000 units S₁ nuclease (Boehringer). For p11-1, the 1-kb *EcoRI* insert was subcloned into pSP64 and the plasmid linearized with *PvuII*. Transcription, hybridization and RNase digestion were performed as described elsewhere⁵², except that 20 μ Ci of ³²P-CTP (760 Ci mol⁻¹) was used in a 10- μ l reaction containing unlabelled CTP at a final concentration of 30 μ M. Protected fragments were analysed on 5% polyacrylamide/7 M urea sequencing gels.

Fig. 2 localized the upstream boundary of exon 1 at ~200 bp 5' of the primer (data not shown). To define this boundary more accurately, we repeated the primer extension analysis with a primer from an even more 5' region (a 91 bp *HaeIII-BamHI* fragment, nucleotides 167-257 in Fig. 2; Fig. 3c). Numerous bands of varying intensity extending 35-165 nucleotides 5' of the primer were observed (Fig. 3a). S₁ nuclease analysis using genomic fragment RB-1 labelled at the *BamHI* site (probe 9 of Fig. 1c; see Fig. 3c) yielded multiple protected fragments corresponding to those observed in the primer extension assay (Fig. 3b), demonstrating that these products represent multiple transcription initiation sites and not strong stops in the reverse transcription reaction. Identical fragments were protected by RNAs from a variety of human neuroblastoma cell lines in which *N-myc* is present in single and multiple copies (not shown), indicating that the heterogeneity of the 5' end of exon 1 is not a result of amplification. Northern blot analysis using probes RR-1 and RR-2 (see Fig. 1a) failed to detect additional coding sequences in the 5' region of the genomic clone (not shown). Thus, exon 1 appears to vary from 450 to almost 600 nucleotides long as a result of multiple sites of transcription initiation.

In summary, these data demonstrate that the *N-myc* gene is encoded by three exons. In support of this conclusion, it is notable that the combined size of the three exons is approximately enough to encode the entire *N-myc* mRNA, and the overall size of the *N-myc* gene (~7 kb) is consistent with the size predicted from transfection studies of the murine gene⁷.

Thus, *N-myc* and *c-myc* appear to have a remarkably similar organization, the most prominent difference being the increased size of exon 3 (1.5 kb for *N-myc* as opposed to 0.9 kb for *c-myc*¹²; Fig. 2).

The apparent similarities in the organization of the *N-* and *c-myc* genes suggest the possibility of additional conserved features. Exon 1 of *c-myc* represents a long, untranslated leader sequence¹³⁻¹⁶. Although the exon 1 of *N-myc* contains two potential translation initiation codons (nucleotides 385 and 521; Fig. 2), termination codons in all three reading frames render it untranslatable. Thus, the *N-myc* and *c-myc* genes appear to have in common a large untranslated first exon (Fig. 2). Furthermore, it has been suggested that regulation of *c-myc* expression is mediated by hairpin-loop structures within the first exon¹³ or between exons 1 and 2 (ref. 15). In this regard, it is notable that numerous potential stem-loop structures with significant standard free energy changes exist between exons 1 and 2 and within exon 2 of *N-myc*, although none is exactly homologous to those found in *c-myc* (see Fig. 2 legend).

As translation of the *c-myc* protein initiates near the 5' end of exon 2 (ref. 12), we searched for a translation initiation codon in the 5' region of exon 2 of *N-myc*. We found three ATGs within this region, at positions 797, 821 and 830 (see Fig. 2), all within the same translational reading frame. From the first ATG within exon 2, an open reading frame continues for 1,392 nucleotides (allowing for proper splicing) before reaching the termination codon TAG at position 2,280. Multiple termination codons interrupt this sequence in the other two translational

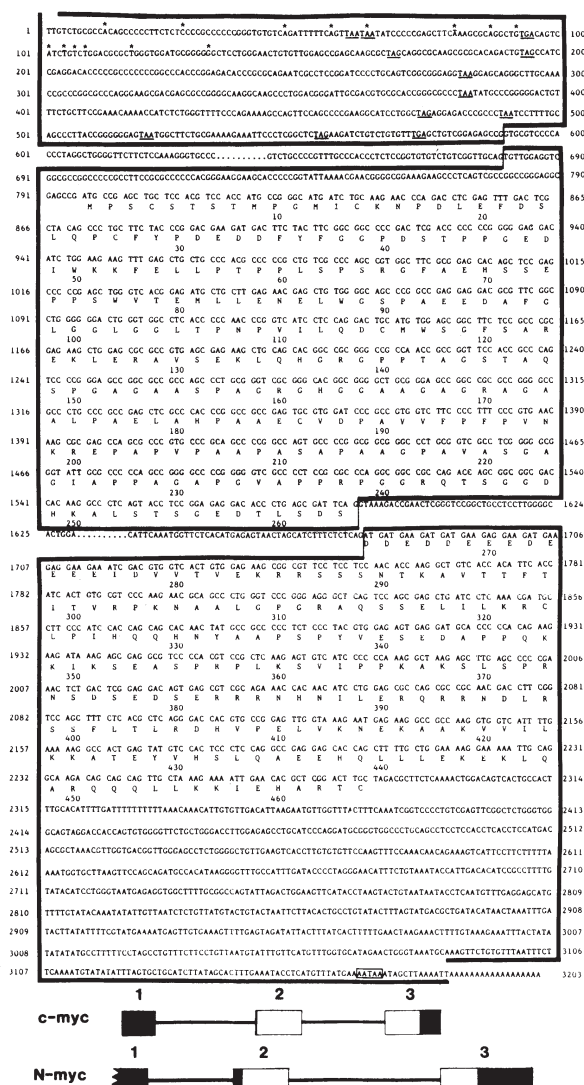


Fig. 2 Nucleotide sequence of human *N-myc*. Restriction fragments were isolated from the cDNA and genomic clones and terminally labelled. Sequencing was by the Maxam and Gilbert⁵³ method using the strategy described in Fig. 1a, b. Boxes around the sequence indicate the positions of the three exons; the open box defining exon 3 indicates that the precise 3' end of the gene has not been determined. However, homology between human and murine genes in this region defines approximately the 3' end of the gene. The open box around exon 1 indicates the heterogeneity of the 5' boundary of this exon. Asterisks indicate transcription initiation sites; the locations of these sites, determined by primer extension and S_1 nuclease analyses, are accurate to within 5 bp. Gaps indicate regions of the introns that were not sequenced. The deduced amino-acid sequence of the putative *N-myc* protein is shown for nucleotides 797–2,279. The adenylation signal (AATAAA) is boxed⁵⁴. Termination codons in exon 1 are underlined. Potential stem-loop structures in mature RNA were identified by computer analyses. The positions and free energy⁵⁵ of the 10 most stable structures are as follows (position indicated by the bases in the stem): 1,274–1,296 versus 1,413–1,439, $\Delta G_0 = -72.2$ kcal mol⁻¹; 211–231 versus 1,269–1,296, $\Delta G_0 = -65.6$ kcal mol⁻¹; 778–808 versus 1,198–1,227, $\Delta G_0 = -55.7$ kcal mol⁻¹; 1,082–1,107 versus 1,230–1,257, $\Delta G_0 = -52.1$ kcal mol⁻¹; 689–715 versus 780–803, $\Delta G_0 = -51.1$ kcal mol⁻¹; 320–345 versus 958–983, $\Delta G_0 = -48.9$ kcal mol⁻¹; 110–141 versus 1,243–1,274, $\Delta G_0 = -47.1$ kcal mol⁻¹; 719–740 versus 1,362–1,387, $\Delta G_0 = -32.3$ kcal mol⁻¹; 799–821 versus 2,525–2,548, $\Delta G_0 = -31.7$ kcal mol⁻¹; 582–697 versus 1,513–1,552, $\Delta G_0 = -29.7$ kcal mol⁻¹. Several of these structures occur in the area of the strong stop to reverse transcription (near the 5' end of cDNA clone p9D, at base 1,403). Additional evidence for secondary structure in exon 2 derives from heteroduplexing experiments and difficulty in sequencing the region. A region of nucleotide sequence homology (60–70%) between the *N-myc* and *r-fos*²¹ and *c-fos*²⁰ genes occurs between nucleotides 1,677 and 1,712. A structural comparison of *N-myc* and *c-myc* is shown at the bottom of the figure. Exons are indicated by boxes: open boxes represent translated sequences, solid boxes represent untranslated sequences. Heterogeneity of the 5' end of *N-myc* is indicated by sawtoothing. The genes are oriented 5'-exon 1-exon 2-exon 3-3'.

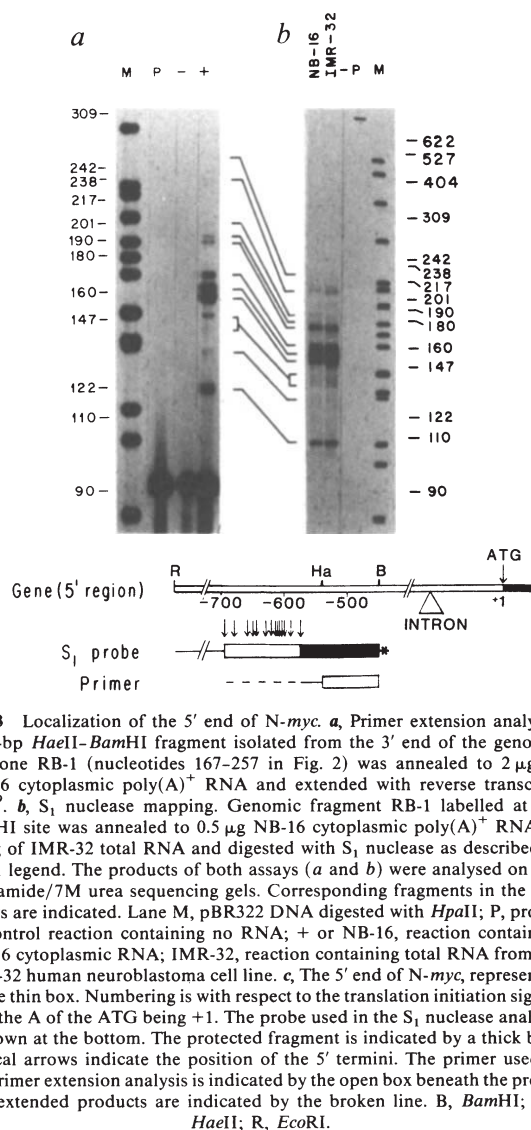


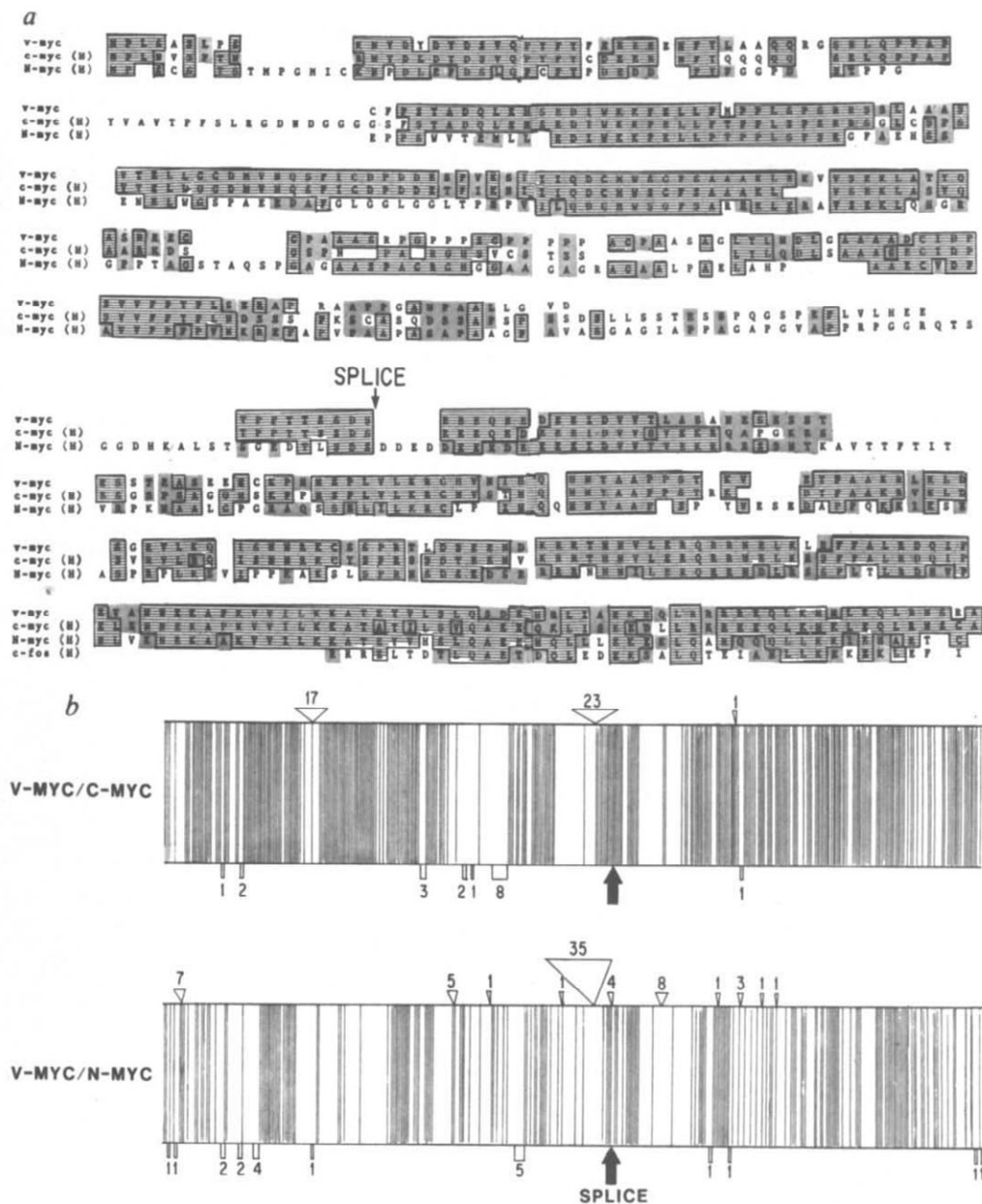
Fig. 3 Localization of the 5' end of *N-myc*. **a**, Primer extension analysis. A 91-bp *HaeIII*-*BamHI* fragment isolated from the 3' end of the genomic subclone RB-1 (nucleotides 167–257 in Fig. 2) was annealed to 2 μ g of NB-16 cytoplasmic poly(A)⁺ RNA and extended with reverse transcriptase⁴⁹. **b**, S_1 nuclease mapping. Genomic fragment RB-1 labelled at the *BamHI* site was annealed to 0.5 μ g NB-16 cytoplasmic poly(A)⁺ RNA or 25 μ g of IMR-32 total RNA and digested with S_1 nuclease as described in Fig. 1 legend. The products of both assays (**a** and **b**) were analysed on 5% acrylamide/7M urea sequencing gels. Corresponding fragments in the two assays are indicated. Lane M, pBR322 DNA digested with *HpaII*; P, probe; –, control reaction containing no RNA; + or NB-16, reaction containing NB-16 cytoplasmic RNA; IMR-32, reaction containing total RNA from the IMR-32 human neuroblastoma cell line. **c**, The 5' end of *N-myc*, represented by the thin box. Numbering is with respect to the translation initiation signal, with the A of the ATG being +1. The probe used in the S_1 nuclease analysis is shown at the bottom. The protected fragment is indicated by a thick box; vertical arrows indicate the position of the 5' termini. The primer used in the primer extension analysis is indicated by the open box beneath the probe. The extended products are indicated by the broken line. B, *BamHI*; Ha, *HaeIII*; R, *EcoRI*.

reading frames. Although we cannot say unequivocally that the ATG at 797 is the *N-myc* translation initiation codon, this seems likely because it is the first ATG of the open reading frame. In addition, this ATG initiation codon and the downstream coding sequence are conserved in murine *N-myc*¹⁷.

Several observations indicate that the proposed *N-myc* coding region is correct, most notably the striking homology of the putative *N-myc* protein to the *c-* and *v-myc* proteins throughout their amino-acid sequences (discussed in detail below). In addition, *in vitro* translation of purified *N-myc* mRNA yielded a diffuse product of relative molecular mass (M_r) 50,000 (50K)–55K (data not shown), a size in accord with the M_r of 50K predicted from the nucleotide sequence of the putative *N-myc* coding region (Fig. 2). Thus, the putative *N-myc* protein is similar in size to the *c-myc* protein; the larger size of the *N-myc* mRNA appears to result primarily from its larger 3'-untranslated region (~900 bp compared with ~350 bp for the *c-myc* mRNA)¹².

Comparison of the deduced amino-acid sequence of the putative *N-myc* protein with that of the human and viral *myc* proteins indicates regions of significant homology in the domains encoded by both exons 2 and 3 (Fig. 4). When the protein products of *N-myc* and *c-myc* are compared with the product of the viral gene, the homology in both cases is higher in the exon 3-encoded domain. In the exon 2-encoded domain, the

Fig. 4 Comparison of the amino-acid sequences of the putative N-myc, c-myc and v-myc proteins. **a**, The amino acids were aligned by inspection, with gaps introduced to achieve maximum homology. Homologous amino acids are boxed and shaded. Amino-acid substitutions judged by McLachlan⁵⁶ to occur at higher than average frequencies are indicated by shading alone. The region of the human *fos* protein²⁰ found in a computer-assisted search of the 3,061 sequences in the NIH protein database (the Protein Identification Resources of the NIH) to be homologous with a region of the putative N-myc gene product is shown. The amino-acid sequence of the c-myc protein is from ref. 12; that of the v-myc protein from ref. 57. **b**, Homology between amino-acid sequences of the v-myc protein and the c-myc and N-myc proteins is shown schematically. The sequence of the v-myc protein was used as a template on which either the N-myc or c-myc protein sequence was superimposed. Homologous amino acids are indicated by a line. Amino-acid insertions in the N-myc and c-myc proteins relative to the v-myc protein are indicated by triangles at the top, deletions by rectangles at the bottom of the grid; the numbers indicate the size, in amino acids, of the insertion or deletion.



homology between v-myc and c-myc is ~65%, that between v-myc and N-myc ~40%; in the exon 3-encoded domain these homologies are ~70% and 50%, respectively. A comparison of the v-myc and N-myc proteins (Fig. 4b) shows that there are clusters of highly conserved amino acids in both coding domains. Within the first protein-coding exon, c-myc diverges significantly from v-myc in three areas, including two large insertions and an 8-amino acid deletion¹². In two of these regions N-myc more closely resembles v-myc; however, N-myc contains a 35-amino-acid insertion in the region where c-myc contains a 23-amino-acid insertion (see Fig. 4). In general, insertions and deletions of amino acids in N-myc relative to v-myc are in regions previously noted to be subject to such structural variations between myc-related proteins¹⁸, in agreement with the notion that these are regions that can tolerate changes in length.

We have demonstrated that the structure of human N-myc resembles that of c-myc (Fig. 2), and that significant nucleotide and amino-acid sequence homology exists between these genes and between their gene products (Fig. 4). The data presented here support the notion that N-myc and c-myc are members of the same cellular proto-oncogene family⁷. c-myc encodes a nuclear protein which has DNA-binding capacity *in vitro*, a

property attributed to the abundance of basic amino acids at the carboxy terminus of this protein¹⁹. This feature is shared by the putative N-myc gene product; thus, the N-myc protein may also be a nuclear, DNA-binding protein. When the deduced amino-acid sequence of the N-myc protein was compared with those of the over 3,000 proteins in the NIH protein database, the most significant region of homology detected (excluding v- and c-myc genes) was between the putative N-myc and c-fos proteins: when residues 425–464 of the predicted N-myc protein are aligned with residues 157–196 of the c-fos protein²⁰, 15 of the 40 amino acids are homologous, with many other conservative substitutions (Fig. 4). In addition, a region of notable nucleotide sequence homology between the N-myc and r-fos²¹ and c-fos²⁰ genes occurs at the beginning of exon 3 (see Fig. 2 legend). Although any significance of these homologies remains to be determined, it is notable that the c-fos protein has also been localized to the nucleus²².

In addition to the structural and functional similarities between the myc genes and their respective products, some regulatory similarities have also been observed, most notably the reduced expression of the N- and c-myc genes in certain tumour lines following chemical differentiation^{23–26}. However,

other aspects of the regulation of the two genes appear to be drastically different. The *c-myc* gene product is expressed in a wide variety of normal and transformed cells where it is probably involved in growth and division^{23,27-30}. In addition, deregulated expression of *c-myc* has been implicated as contributing to the development of numerous classes of tumours³¹⁻³⁵. However, expression of *N-myc* is detectable in a very restricted set of normal or transformed cells and deregulated expression of *N-myc* has been implicated only in a limited set of tumours^{9,10}. Neither the control nor the function of the differences in expression of the two *myc* genes is understood. However, it is notable that the promoter regions of the two genes appear to differ. Whereas transcription of *c-myc* mRNA initiates at two cap sites located ~150 bp from one another, each of which is preceded by a classical TATA motif¹³⁻¹⁶, transcription of *N-myc* mRNA appears to initiate at multiple sites, most of which occur in the absence of an upstream TATA-like sequence. Such initiation heterogeneity has also been observed for the dihydrofolate reductase³⁶ and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase)³⁷ genes and for the early and late regions of simian virus 40 (SV40)^{38,39}. Significantly, sequences upstream of the major *N-myc* initiation sites show several similarities to the promoter of SV40. This region is G+C-rich (~70% G+C) and contains a 9/10-nucleotide match with the imperfect 21-bp repeat of the SV40 promoter, including the hexanucleotide CCGCCC, which is important in transcription of the viral early and late genes⁴⁰⁻⁴². This 'GC' motif has also been observed in the promoter of the herpes thymidine kinase gene⁴³ and in the control region of several housekeeping genes including HMG CoA reductase³⁷. An additional property that the HMG CoA reductase³⁷ gene has in common with *N-myc* and *c-myc*¹³⁻¹⁶ is the presence of an exceptionally long 5'-untranslated leader sequence, most of which derives from a 5' noncoding exon. The strong conservation of the 5' noncoding exon between the *c-myc* genes of mouse and human suggests a significant functional role for this exon: one proposed function is a regulatory role with respect to *c-myc* expression^{13,15,31}. Although no homology is detectable between the 5' exon of *N-myc* and that of *c-myc*, the 5' exons of human and murine *N-myc* show 85% homology (not including insertions and deletions)¹⁷; this evolutionary conservation of the 5' exon of *N-myc* also suggests a significant functional role for the exon (in analogy to *c-myc* this may be a regulatory role). If so, the lack of homology between the 5' exons of *N-myc* and *c-myc* would be consistent with divergent regulatory patterns of the two genes.

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MATTERS ARISING

Mapping of general anaesthetic target sites

FRANKS and Lieb¹ have suggested that the comparative effectiveness as general anaesthetics of members of the *n*-alkane and *n*-alkanol homologous series may be understood in terms of their effects on the activity of a water-soluble protein, firefly luciferase. In particular, they propose the following: "When general anaesthetic target sites in animals and the luciferase protein are mapped out using the fine details of the potency data, remarkable similarities are revealed". We disagree. Consider first the *n*-alkane results. Figure 2 of Franks and Lieb indicates that roughly equal aqueous concentrations of *n*-pentane, *n*-hexane, *n*-heptane and *n*-

octane are required for 50% inhibition of luciferase activity. However, there are data available on the effectiveness of these compounds as general anaesthetics. The data of Fuhner (see Table 4 of Ferguson²) and Crisp *et al.*³ on alkane-induced narcosis in mice and barnacle larvae respectively show very clearly (given knowledge of the saturated aqueous concentrations⁴) that in both these widely disparate species, the effective aqueous concentration decreases progressively from *n*-pentane to *n*-octane; this is shown here in Fig. 1a. Thus alkane activity as a general anaesthetic and as an inhibitor of firefly luciferase could be said not to correlate at all.

For the *n*-alkanol system, Franks and Lieb compare luciferase data with their chosen combination of data from the

studies of Vernon⁵ and Meyer and Hemmi⁶ on anaesthesia in tadpoles. These three sets of results are plotted in their original form in Fig. 1b (the plots have been displaced from each other vertically so as to show more clearly the variations in slope which are to be compared). The data of Meyer and Hemmi show no correlation with the luciferase results. The usefulness of Vernon's much earlier results is marred by the fact that, for the C₅ experiment, Vernon used not *n*-pentanol but an isopentanol of unspecified structure (perhaps even a mixture of the two isopentanol) and arbitrarily divided his result by two. The correct position of this point (shown in parentheses in Fig. 1b) is thus unclear, a feature which Franks and Lieb do not mention. Given also that Vernon says nothing about the purity of his

n-alkanols—which, in 1913, was probably not very high—such correlation as might be claimed between the luciferase data and

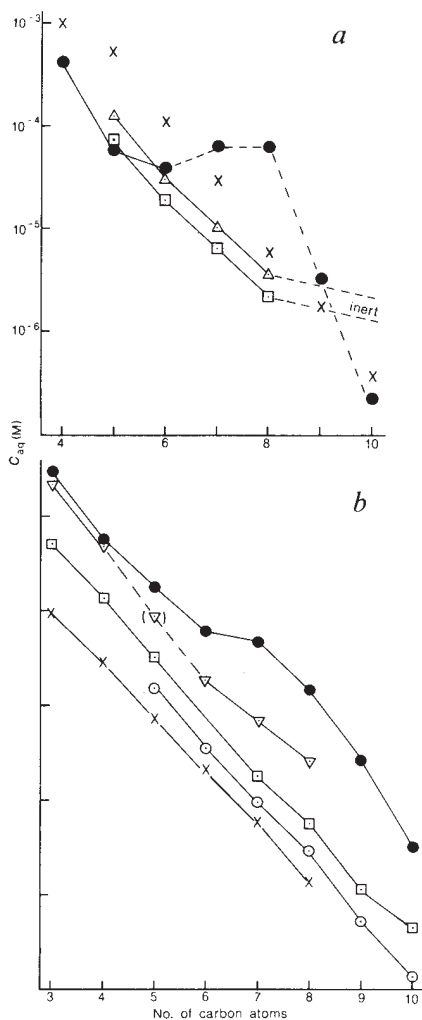


Fig. 1 The dependence on chain length of aqueous concentrations of *n*-alkanes (*a*) and *n*-alkanols (*b*) required to produce a given endpoint in various systems. The abscissa shows the number of carbon atoms in the molecule. The ordinate for *a* gives the aqueous concentrations (C_{aq}) of *n*-alkane required to produce a saturated solution in pure water¹ (x); inhibit luciferase activity by 50%¹ (●); induce narcosis in mice² (Δ), and induce narcosis in barnacle larvae³ (□). The data for mice are given as the concentration in Ringer's solution at 20 °C which would be in equilibrium with the blood of the mouse. The ordinate for *b* again gives the required concentrations but the plots for each system have been displaced vertically to an arbitrary extent in order that the slopes may be more readily compared. The systems are: ●, 50% reduction of luciferase activity¹; ▽, reversible suppression of spontaneous movement in tadpoles (the point in parentheses is derived from data for an undefined iso-derivative)⁵; □, reversible suppression of reflex movement in tadpoles⁶; ○, 50% reduction of Na current in a voltage-clamped squid giant axon⁷; x, reversible inhibition of luminescence in bacteria⁸.

Vernon's results is, to say the least, unconvincing, and does not justify Franks and Lieb's Fig. 3.

While the relevance to general anaesthesia is debatable, similar experiments examining the effects of alcohols on the sodium current of the squid giant axon⁷ and on bacterial luminescence⁸ (Fig. 1*b*) show no significant irregularities in the range pentanol to heptanol and exhibit clear similarities to the data of Meyer and Hemmi on anaesthesia in tadpoles.

For the two families of *n*-alkanes and *n*-alkanols there remains the one similarity that, for the alkanols, the anaesthetic potency and the binding to luciferase decline beyond *n*-dodecanol. But there is thought to be little luciferase in the central nervous system.

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FRANKS AND LIEB REPLY—We disagree with the criticisms of Elliott and Haydon. The purpose of our paper¹ was to demonstrate that the abrupt cut-offs in general anaesthetic potencies observed when ascending homologous series of *n*-alkanes and *n*-alcohols can be mimicked by a pure soluble protein, thus lending further support to the idea that the primary target sites in general anaesthesia are proteins. These cut-offs occur at very different positions in the alkane and alcohol series, when anaesthetic ED_{50} (50% effective dose) concentrations become greater than saturated aqueous concentrations.

For the *n*-alkanes, we found¹ that the luciferase cut-off occurs between C_6 and C_7 . In animals, the cut-off point is species-dependent¹, occurring between C_6 and C_{10} . In view of the different cut-off positions, it is quite wrong to expect a point-to-point correlation in potency data in this transition region: Elliott and Haydon are comparing (luciferase) data just past the cut-off with (animal) data just before the cut-off. They have also ignored data for fish, which show a cut-off very similar to that for luciferase¹. What is important is that the luciferase enzyme exhibits an alkane cut-off at a position similar to those observed in animals.

For the *n*-alcohols, we found that, in common with general anaesthesia, the luciferase cut-off occurs much later than

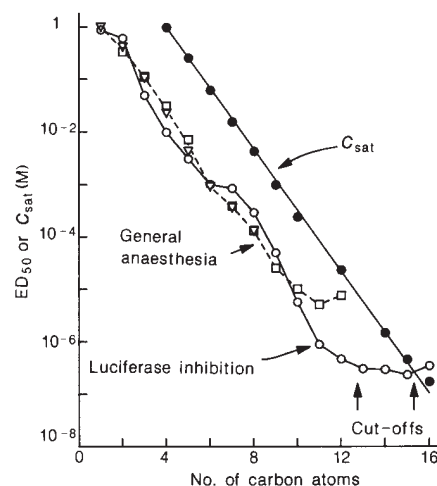


Fig. 2 The effectiveness of the *n*-alcohols as general anaesthetics and as inhibitors of firefly luciferase. ○, ED_{50} s for luciferase¹; ▽ and □, general anaesthesia ED_{50} s for tadpoles, from refs 2 and 3, respectively; ●, maximum aqueous concentrations (solubilities)⁴.

it does for the *n*-alkanes and this is due to a levelling-off of ED_{50} s for the higher alcohols. These points are clear from Fig. 2, where data for general anaesthesia and luciferase inhibition are compared directly. (Elliott and Haydon have obscured these points by choosing to plot only data between C_3 and C_{10} and by offsetting their curves on a relative scale. Also, their inclusion of data for bacteria and squid axons seems to us a red herring.) The close correspondence between the data of Vernon² and those of Meyer and Hemmi³ shows that the worries of Elliott and Haydon are unfounded. As for the inflection at about C_6 , it is clear that, although small, it is a real feature of the combined general anaesthesia data. (Elliott and Haydon's concern about Vernon's C_5 data point is not relevant, as the same feature is found even if this is disregarded.) However, this minor feature has no bearing on the mechanism of the alcohol cut-offs, which are due to the much larger changes in slope at about C_{11} .

In summary, we feel that, in contrast to lipid bilayers, protein pockets similar to that found on firefly luciferase provide both a plausible and an economic explanation of cut-off effects in general anaesthesia. Despite our disagreements with Elliott and Haydon, we can at least concur with their final sentence!

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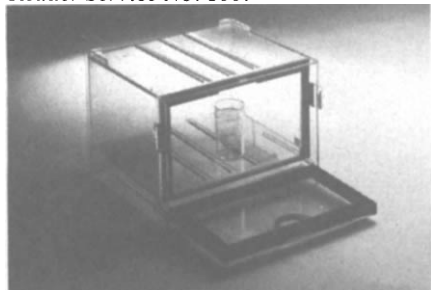
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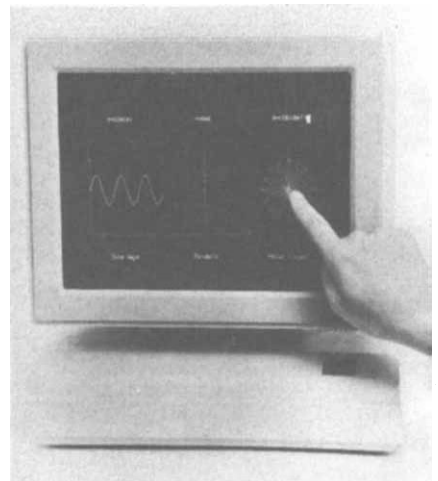
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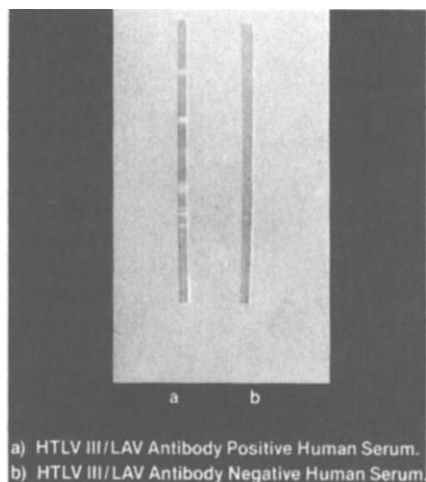
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Reader Service No. 105.

These notes are based on information provided by the manufacturers. To obtain further details about these products use the reader service card bound inside the journal.

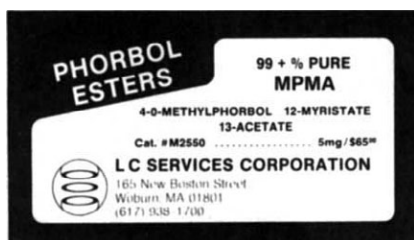
• A rapid and simple new radioimmunoassay kit for measuring rat atriopeptin III and other rat atriopeptins has been introduced by Upjohn Diagnostics. The simple four-step procedure provides results in a day and a half, and is available in a variety of formats for different levels of sensitivity. The assay antibody cross-reacts with atriopeptins I and II and rat alpha-atrial natriuretic peptide. *Reader Service No. 106.*



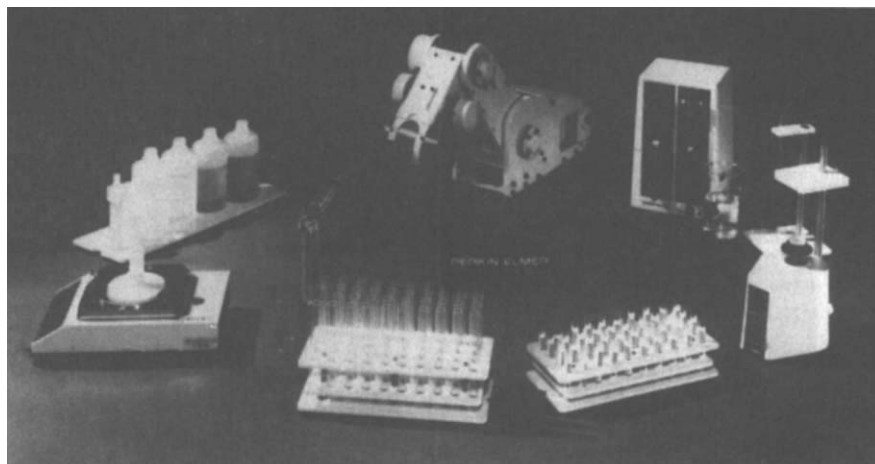
• Bio-Rad has introduced a range of products for AIDS Western blotting, a highly specific and sensitive technique for the serological analysis of HTLV-III/LAV virus and characterization of viral gene products. The enzyme-linked immunoelectrotransfer blot technique (EITB), developed at the Center for Disease Control, Atlanta, USA is a highly standardized method, utilizing high field intensity Western blotting after electrophoresis of viral proteins in SDS-PAGE. Two systems are available: one for rapid separation using miniature gels, the other provides high capacity, high resolution separations on standard gels. *Reader Service No. 107.*

• Laserspec, from Spectrolab, is a new, very high transmission tunable filter designed specifically for lasers. It interfaces directly with any laser and is suitable for beam widths of 0.5–5 mm diameter. Overall specifications are 80% transmission, 2 Å bandwidth, 170 nm – 4 µm wavelength range. Being continuously tunable, Laserspec can be used in laser spectroscopy, Raman, and wavelength rejection. *Reader Service No. 108.*

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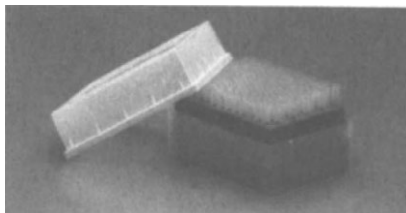
Circle No. 53 on Reader Service Card.



The Perkin-Elmer robot surrounded by its potential targets.

• The MasterLab robotics system from Perkin-Elmer provides the missing link in the entirely automated and integrated analytical laboratory: automatic sample preparation. The basic MasterLab system comprises a robot, a robot electronics module, a communications interface and an IBM PC. Each system can be designed to meet the automation requirements of a specific application or sample preparation procedure. Depending on the application, accessory modules such as mixers, dispensers or balances may be required to interface with the system. Several levels of automation are possible with the MasterLab system, for example: total automation which integrates robotic sample preparation with analysis and a laboratory information management system; sample preparation for subsequent transfer to instruments for analysis; specific applications, such as weighing samples, filtering, dispensing or handling dangerous substances. *Reader Service No. 109.*

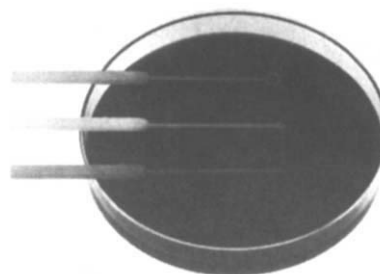
• The latest freeze dryer from Martin Christ GmbH is a unit in which the ice condenser is integrated with the drying chamber. During the drying process the water vapour is frozen, taking the shortest way over the ice condenser surfaces which can be cooled to -75°C , and are sited right and left of the product shelves. The drying period can be reduced by 30% compared with conventional devices, where the drying chamber is separated from the ice condenser compartment. *Reader Service No. 110.*



A rack of 125 pipette tips from Robbins.

• The disposable Titer-Pak II, from Robbins Scientific, consists of a box with telescoping cover and a sturdy, non-flex rack pre-loaded with 125 pipette tips. The tips

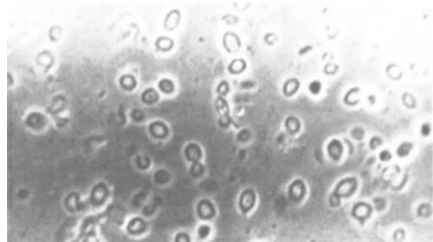
are made of virgin polypropylene and can be used with Pipetman, Eppendorf and Excalibur pipettors up to 1,000 µl. This entire unit is disposable or can be autoclaved repeatedly without any distortion resulting. It is also available sterilized by cobalt irradiation. *Reader Service No. 111.*



Disposable 'Bioloops' come ready-sterilized.

• Elkay disposable Bioloop inoculating loops and needles are now available in a choice of four sterile packaging options: individually wrapped, 10-pack, 30-pack and 50-pack. Bioloops are pre-sterilized so there is no need for flaming prior to use, reducing risk of spreading pathogenic aerosols and saving time. Bioloops are available with a needle at one end and a 10 µl or 1 µl loop at the other. The third type has both a 10 µl and 1 µl loop. Each model is colour-coded for easy identification. *Reader Service No. 112.*

• New from Jasco Inc. is a research-grade spectrofluorometer, the Model FP-770. The instrument features $f/2.5$ optics permitting scanning from 200 to 850 nm (optional) at 1,000 nm per min. A computer data station for all user interaction provides functional keys for extreme ease of operation. The dedicated software program with its built-in self-diagnostic capability offers a 19-function range of computerized luminescence spectroscopy. The FP-770, a dual-beam with reference photomultiplier system corrected for light source fluctuation, has a wavelength accuracy of ± 1.5 nm and repeatability of ± 0.3 nm. *Reader Service No. 113.*



Damage is kept to a minimum by the Microfluidics continuous-flow cell disruption system.

• Controllable continuous-flow cell disruption with a minimum of damage to cell contents is achieved with the Microfluidics M-110 B. Self-contained, compact and light-weight, the M-110 B can be run on an open bench or placed in a laminar flow hood if required. The unit provides the option of processing dilute or concentrated cell suspensions as small as 10 ml through to 1 litre per minute pilot production runs. The M-110 B temperature-controlled models can operate down to -25°C , and full-scale models up to 20 gallons per minute are available.

Reader Service No. 114.



A program for the determination of dry weight and moisture content is available from Mettler.

• The dry weight and moisture content of samples taken from the drying oven can now be determined quickly and easily with a new program from Mettler LabWare—DRYING. It is stored on a microcassette and used as application-specific software in combination with a hand-held Epson HX-20 computer and a Mettler analytical or precision balance. DRYING stores the weight values and codes of up to 300 samples. After drying in the drying chamber, the samples are identified through their codes during back-weighing and the desired values are determined automatically. DRYING automatically records every determination. In the case of a whole sample series, it even determines the mean value. The program uses a dialogue to guide the operator through the determination, and instructions appear on the 4-line computer display in English, French or German, or any desired fourth language.

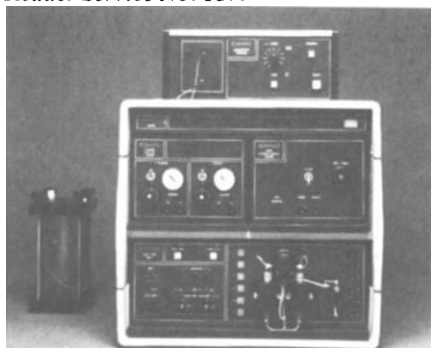
Reader Service No. 115.

• Two different designs are available in the Nordia fume cupboard range. The fume cupboards are fitted with toughened glass in a variety of timber and metal fabrications. The Nimrod range of aerodynamically designed compensated airflow fume cupboards, complying with the performance and safety requirements of DD80, is available in four widths. Standard features of the Nimrod range include a control panel incorporating a key-operated panel isolator with remote push button station for the extract fan motor, showing visual motor running/failed conditions. Other safety features include an optional audio-visual sash high and airflow failure alarms, a sash locking system and a heat sensor alarm/extinguisher. The economical Harrier fume cupboard range has been designed primarily for work involving lower levels of toxicity. Mason Nordia also provides and installs fume extraction systems, mechanical and electrical services and special gas installations.

Reader Service No. 116.

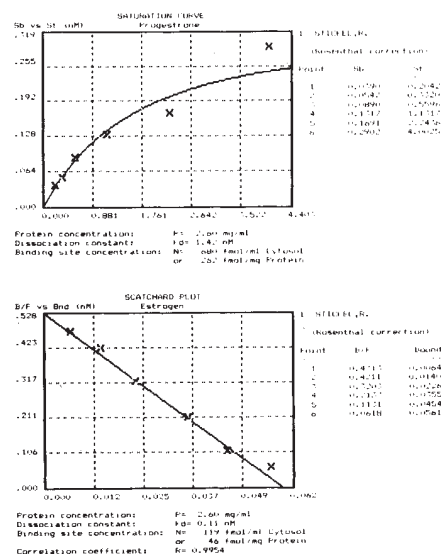
• Two new columns for the analysis of amino acids by ion chromatography are now available from Dionex. The HPIC-CS4 cation exchange and the HPIC-AS8 anion exchange columns provide complementary selectivities for the confirmation of amino acids, using advanced pellicular resin technology which allows high efficiency and high-speed separations without the limitations of traditional, totally porous ion exchange columns. The columns provide the specificity of classical ion exchange and the speed of pre-column derivatization. The Dionex ion chromatograph for amino acids is ideally suited for the analysis of amino acids in complex matrices with minimal sample pretreatment. The same system can perform state-of-the-art ion chromatography including the analysis of other ionic organic compounds and an extensive range of inorganic ions.

Reader Service No. 117.



A complete amino-acid chromatography system from Dionex.

• Packard Instrument Company has developed a new software program, COMBICEPT, for steroid receptor assays. Using a single assay, COMBICEPT provides simultaneous calculations of the oestrogen and progesterone receptor concentrations in tissue cytosols. Designed as part of Pack-



Packard's latest steroid receptor assay program at work.

ard's Personal Computer Data Acquisition and Analysis System (PC-DAAS), COMBICEPT provides simultaneous dual-label receptor assay calculations (oestrogen and progesterone), on-screen curve editing, multi-assay, multi-tissue data reduction, and on-line/off-line processing. Receptor data are stored on a floppy disk, and up to nine assay protocols can be stored. Each tissue is identified by a name, and the final results calculated using the individual protein concentrations. The software can be implemented on all present production models of Packard Tri-Carb and Auto-Gamma counters. Data for other systems can be processed manually.

Reader Service No. 118.

• AMOR, from Spark of Holland is a new AMperometric detector for HPLC. This new electrochemical detector features a solid-state *in situ* reference electrode which is entirely maintenance-free. The working electrode is constructed so that the edge of the glassy carbon material is extraneous to the cell. The edge of the working electrode often causes noises and drift problems in EC detection. The cell is thermally insulated and electrically shielded. The cell volume may be varied by applying different (Teflon) spacers (0.4 – 1.0 μl). Sensitivity range is from 0.1 nA to 1000 nA, and potential setting from -2 to $+2\text{V}$.

Reader Service No. 119.

• CelsiDot single level temperature sensitive labels are now available on rolls with 1,200 pieces of identical dots. There are 40 different temperature ratings available between 40°C (105°F) and 260°C (550°F). The white temperature sensitive indicating triangle turns permanently black when exposed above its rated temperature level. The self-adhesive labels can be applied to any clean, dry surface.

Reader Service No. 120.

The brain drain is here again

from Richard Pearson

Much is made of the 'brain drain' of UK scientists to overseas posts, but little is known about the extent of the problem.

THE brain drain is back in the news in the United Kingdom. The Civil Service is trying to plug the leakage of computer scientists to industry. The UK scientific community is worried about losses overseas. The universities complain about losses of academic lecturers to industry. There are some hopes that the Strategic Defense Initiative will reduce the brain drain from the United Kingdom. But in Ireland emigration remains an emotive subject with some educationalists seeking to play down talk about a brain drain. It is clear from these recent examples that the term 'brain drain' itself embraces a number of different flows and any assessment of its scale or impact depends on the perspective of the reporter.

Last year the Advisory Board for the Research Councils (ABRC) reported to the government its growing concern about the increasing losses of talented British scientists overseas. Its subsequent detailed enquiry, based on 40 leading research groups, showed that departments were gravely concerned about the losses not only of their most talented students and postdoctoral research workers but also of outstanding senior scientists. However, because the departments usually only employed small numbers of scientists they found it hard to present a statistical assessment of the flow or of the trends over time. They did, however, note a growing loss of senior chemists, materials scientists, biochemists and molecular biologists. The physicists and to a lesser extent the engineers reported far fewer problems. ABRC was able to identify 45 British senior scientists working overseas.

A more detailed study of biotechnology two years ago (*Nature* 309, 654; 1984) revealed an estimated 250 UK nationals who had gone overseas in the previous decade, a total which excluded newly qualifying postgraduates¹. The biotechnologists had gone to a wide range of countries and employing sectors. Typically leavers were aged 26–30 although one in three was aged over 30. The leavers went from industry, higher education and research institutes. The majority went however to jobs with commercial organizations. Nearly half went to the United States, with Switzerland and other European destinations for another one in three. In all, UK personnel went to 13 different countries and were working for over 65 different organizations. One new overseas

biotechnology venture company had recruited over 16 UK nationals.

The reasons for losses overseas have been more related to improved job prospects and research opportunities overseas than salary, although the latter has usually come as a healthy bonus, and can be a major barrier for those thinking about returning. ABRC reported a growing frustration by senior scientists over the improved level of facilities, both recurrent support and equipment, available overseas, and the frustration over the difficulties in getting research funding approved in the United Kingdom. For younger scientists and those newly qualifying, then the flow overseas is seen as part of a more regular pattern of broadening their experience, usually in the United States, although growing numbers are thought to be going because of the lack of jobs in the United Kingdom. The recent restrictions on work permits in the United States may be restricting the scale of this potential outflow. The 'New Blood' scheme designed by the University Grants Committee (UGC) was thought to be helpful in the latter case, while additional UGC research funding is helpful with the former. The government has also cited the opportunities for 'star wars' research now available in the United Kingdom as another inducement to stem the flow. Two other factors aggravating the flow overseas are seen to be aggressive recruiting in the United Kingdom on the part of US universities and industry, and the greater receptiveness of US industry to novel and untried ideas. Where British laboratories have world class facilities, as at the Science and Engineering Research Council Nuclear Structure Facility or the Cambridge Laboratory of Molecular Biology, then they seem able to attract staff back from overseas and losses are not a significant problem. However, growing concern is reported over the level of funding and the lowering of morale which can only encourage further losses in the future.

Losses overseas are, however, usually

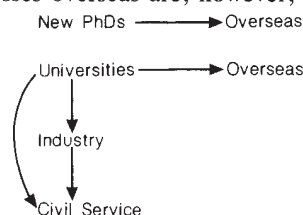


Fig. 1 The brain drain — who flows where?

only a very small part of mobility between sectors and its direct effect has been hard to evaluate. Rather it has been seen more as a loss of potential. ABRC also reports growing concern about losses from academia to industry, particularly of chemists. However, one person's loss is another's gain and flows between sectors within a country have not tended to be classified as a 'brain drain' in the past. A common international problem in times of skill shortages is that there will normally be a flow from academia to industry, the latter usually being quicker to respond to staff shortages by raising salaries and improving conditions. Any shortage of academic, and more specifically, teaching staff will however undermine the future supply of suitably qualified graduates and so reinforce the longer-term impact of shortages.

Ironically, while ABRC was reporting concern about the universities' inability to compete with employers in industry and the scientific civil service, the latter, in the information technology area, is increasingly suffering from an outflow of its specialist staff to industry.

The Civil Service has historically trained all its own computer staff, rarely, if ever, recruiting experienced staff from the open market. Information technology is now being increasingly used in the Civil Service, pushing up the number of skilled staff needed, while at the same time official policy has been to hold down the rate of growth in public sector pay levels. As a result there has been an increasing flow out of the Civil Service into better-paid jobs in industry and commerce. Responding to the growing problems, the government and the trades unions have now agreed increases in pay levels in key grades of up to 26% to help stem this brain drain.

There are many dimensions to the brain drain, the range and direction of 'mobility' covered widening as concern about skill shortages and funding has increased. Quantification in this complex area is rarely attempted, more qualitative assessments and key names are usually the prime ammunition of those seeking to influence policy. □

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1. Pearson, R. & Parsons, D. *The Biotechnology Brain Drain* (Science and Engineering Research Council, 1984).